UNIVERSIDAD AUTÓNOMA DE MADRID
DEPARTAMENTO DE BIOQUÍMICA

IMPLICACIÓN DE LAS PROTEÍNAS DE RESPUESTA AL ESTRÉS EN ATEROTROMBOSIS

Julio Madrigal Matute

Madrid, 2012

DEPARTAMENTO DE BIOQUÍMICA
FACULTAD DE MEDICINA
UNIVERSIDAD AUTÓNOMA DE MADRID

IMPLICACIÓN DE LAS PROTEÍNAS DE RESPUESTA AL ESTRÉS EN ATEROTROMBOSIS

Memoria que presenta el licenciado en Biología

Julio Madrigal Matute para optar al grado de Doctor

por la Universidad Autónoma de Madrid

Directores de Tesis:

Dr José Luis Martín Ventura

Dr Jesús Egido de los Ríos

Instituto de Investigaciones Sanitarias Fundación Jiménez Díaz, Universidad Autónoma de Madrid

Don José Luis Martín Ventura, Doctor en Ciencias Químicas, y Don Jesús Egido de los Ríos, Catedrático de Medicina de la Universidad Autónoma de Madrid,

CERTIFICAN

Que Don Julio Madrigal Matute, Licenciado en Ciencias Biológicas por la Universidad Complutense de Madrid, ha realizado bajo su dirección el trabajo titulado "Implicación de las proteínas de respuesta al estrés en aterotrombosis" que presenta como Tesis Doctoral para alcanzar el grado de Doctor por la Universidad Autónoma de Madrid.

Y para que conste, firmamos la presente en Madrid a 26 de Abril de 2012

Los directores de tesis, Vº Bº El tutor,

Dr. José Luis Martín Ventura Dr. Jesús Egido de los Ríos Dr. Francisco Portillo

Este trabajo ha podido llevarse a cabo gracias a la financiación de los proyectos del Ministerio de Ciencia y Tecnología (SAF2007/63648 y SAF 2010-21852), Fundación Ramón Areces, CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (PI10/00072) y la Red de Trabajo Europea European (HEALTH F2-2008-200647) y la Sociedad Española de Aterosclerosis.

A mi mami

"El maleficio hispánico ha sido deshecho; muy duradero y cerril habrá de ser un gobierno inculto para que deje perecer en su país esa vegetación intelectual que ya se propaga, y se perpetuará por sí misma si se cuida y estimula."

Julio Rey Pastor [(Logroño (España) 1888 – Buenos Aires (Argentina) 1962].

Agradecimientos

Aunque sé que no le gusta que lo diga en alto, mi madre siempre dice aquel refrán; "es de bien nacido ser agradecido". No es difícil imaginar que esta sección va en esa línea, aunque cierto es que no debería hacer falta esta sección para que alguien que haya trabajado a tu lado durante los últimos 5 años sepa que le estás agradecido, ni para la familia o amigos más cercanos, pero no deja de ser bonito este apartado, ¿o no?. De hecho, esta sección no deja de ser el capítulo de la tesis más injusto, por dos motivos fundamentales; el primero es que siempre hay alguno que queda disgustado con esta sección, bien aparece tarde o poco o bien no lo hace por descuido o por un lastimoso olvido… En segundo lugar es injusta porque pasas tus últimos 5 años trabajando en algo que se plasma en algo más del centenar de hojas de 100 g, muy interesantes todas y cada una de ellas, con dibujitos y todo, y al final la gente sólo se lee esta parte. Esta sección sólo debería poder leerse tras la lectura obligada, con interés y concienzuda de toda la tesis, pero por no ponernos a innovar a estas alturas seguiremos el formato clásico.

Así que debo empezar dando las gracias al Dr. Egido, ya que sin su dirección y su esfuerzo por hacer de este laboratorio lo que es, nunca habría llegado tan lejos.

A Laura por su paciencia infinita y hacernos a todos el trabajo un poquito más fácil.

Por orden de aparición debería seguir con Pepe y creo que lo menos que se puede decir es que no sólo tengo un gran director de tesis sino un gran amigo, y ¿al final qué es lo que nos queda en la vida cuando nos quitamos lo superfluo? Sólo espero haberte devuelto durante este tiempo toda, o al menos en parte, la confianza que pusiste en mí, en un gualdrabas cagonet! Espero que esos intangibles de los que tanto hablamos nos guíen bien y hacia el mismo lugar algún día.

Para continuar en estricto orden de aparición debería seguir con mi último y más longevo (no en edad sino en tiempo compartido) compañero de poyata, el hombre que menos sabe de baloncesto del mundo, pero no así de ciencia. Gracias a lo que sea escogiste el segundo camino porque con el primero, no sé yo… Es un placer compartir cervezas, charlas, risas y de vez en cuando, aunque muy de vez en cuando algo de ciencia contigo Luisete.

Aquí ya el orden de aparición se rompe por completo y esta sección se guiará por la total anarquía de mi orden imaginario. Así que me gustaría continuar con la Jefa con la que es un placer compartir cualquier momento en el trabajo o fuera de él, bondadosa como pocas, realmente eres un ejemplo en todos los sentidos y una científica superlativa.

Mi pixyta, ¿qué puedo decir que no sepas ya? Esto suena a balada de amor así que no continuaré por esos derroteros, pero sabes que es verdad que es amor aunque otros lo quieran llamar otra cosa. Cuántas risas, chascarrillos, incluso chirigotas (las mínimas) compartimos; lavándonos los dientes, en el microondas, en tu sitio ese de la esquina en el que te marginas… ¿Qué mejor terapia para cualquier adversidad que el humor? Mucha suerte ha tenido el recién llegado Andresito (Iniesta) con un padre tan "exquisito".

Beñako, Bernardo, Boñat, Iñaki o Señorita Beñat entre otros, cualquier nombre me sirve para darte las gracias porque si algo se sabe cuando se curra contigo es tu ilimitada capacidad para ayudar al que está al lado sin pedir nada a cambio, sin una mala cara, sin un "tengo prisa, date vida". Además de darte las gracias por el hecho de que un día se te cruzó por la mente de que podría ser una buena idea de que yo trabajase aquí.

Esto se alarga más que lo esperado, y por desgracia no ha hecho nada más que empezar, así que a tiempo estáis de cerrar el libro…

No puedo olvidarme de los que se embarcaron hacia destinos lejanos y que me ayudaron a dar los primeros pasitos en este labo; Melany, la Jefa-Bego con su pequeñita Ania que me ayudó a todo en los primeros días, el Dr Kyxu con los ratones y nuestras charlas a altas horas de la noche sólo acompañados por Carmen (que se merece un apartado para ella solita), la Dra Ortiz-Muñoz siempre ayudando en cualquier cosa que se necesitase o el Dr Rodríguez-Vita que siempre me palicea al Catán/Colonos. Hablando de juegos de mesa no puedo olvidarme del gran Charlie, siento haber sido tan cateto a la hora de aprenderme las reglas, pero ya sabes que a mí el primer cl de cerveza me nubla las pocas neuronas que tengo y a Beíta, cuántos buenos momentos en el labo, pasando frío con el agua heladora de la piscina… y sobre todo gracias por no darme capones y cedérselos todos al gran cuello de Avo.

También me gustaría agradecer a todos los que trabajaron durante este tiempo un poquito más cerca como Olalla, Yolanda a la que debo una gran parte de este tesis y a la que me hubiese encantado tenerle que agradecer mucha mayor parte de este tesis, espero que te vaya todo muy bien porque te lo mereces, Pris, Patri muchas gracias por tu esfuerzo en todos los sentidos y sobretodo en entender mi letra y mi desorden mental a la hora de organizar las cosas, incluidas las cajas del -20 y -80 y Tarín por descubrirme

que existen las bolsas de trasporte de tupper marca Apple y por tu risa de cada día, aunque sea la del malo de la película. A la Roxi mucha suerte en tu aventura francesa, aunque no creo que la necesites porque con tal encanto de persona y tan buena compi de curro la suerte es de los que te acogen. A Valva por su hincapié en que las cosas salgan por complicadas que parezcan, ese luminómetro encantador verdad??? Los llegados hace menos tiempo como Ernesto y Elena, gracias por vuestro esfuerzo y ganas por aprender, espero que os haya podido ayudar aunque sea un poquito a que la llegada a un sitio nuevo haya sido más fácil. Una persona que ha sido también muy importante en esta tesis ha sido Oli, muchas gracias por tu ayuda cuando estuve allí y por todo tu apoyo mientras he estado aquí, espero que nuestros caminos se sigan cruzando por muchos años.

Cómo olvidarme del grupo de la tarde, formado por Conchi (o Concha como descubriría más adelante y que nunca me acostumbraré a llamarte), Avo y Alberto (o Adalberto). Parece mentira que a medida que el atardecer iba anunciando el final del día, estuviese a punto de llegar uno de los mejores momentos del mismo; 19:00, Frankie girando en la cedetera, primeros compases y el tío Frankie indicando de manera incansable día tras día en aquella segunda pista el camino, tienes toda la razón "*I* also *want to wake up, in* the *city that never sleeps*". Cuando Frankie susurraba eso en los oídos de toda la 4ª planta, puesto que no lo hacía a un volumen bajito, empezaban las bromas que sin "Sergio Paulo Barbosa Valente" (duda) hacían que nos volviésemos un poco loco Ghatti no Avo (apodo que no puedo tener peor rima)ʔ. Siento no haber podido aprender mucho más de tus clases de fútbol pero aparte de imitar a RGB poco puedo hacer ya, aunque en la informática dimos el do (re, mi, fa…) de pectoral. Concepción hemos pasado horas de estrés a las mil, huracanes en el Bronx, y sobre todo muy buenos momentos, muchas cenkius por todo! Sr Alperto pues lo que me surge de dentro es decirte que echo mucho de menos y con eso creo que poco más se puede decir. Ha sido un placer tan inmenso compartir estos años no sólo de trabajo sino de amistad, que el día que me dijiste que te ibas en 2 días del labo me entró una tristeza tal que es difícil plasmar aquí en 3 líneas escritas sin estilo alguno, mucha suerte en vuestra nueva aventura. Espero que esa idea que se propuso del congreso imitación al de Ámsterdam se haga algún día realidad.

A la Cristi Chunfli porque aunque a veces consigas acabar con mi infinita paciencia, es muy ameno compartir curro contigo! A la Loli (Chama) por todas sus risas e historietas increíbles que nos han hecho pasarlo tan bien en tantas sobremesas, a Anita por su dulzura, A la Su porque me ha enseñado un montón de cosas del labo, ayudado siempre que ha podido y aunque se ha llevado alguna moussaka de recompensa… siempre está ahí para echar una mano a quien lo necesite.

A los que han llegado hace menos pero que no esperaron mucho para no perderse su primera SEA, verdad Ainhoaʔ, menos mal que el boli sale con agua y jabón… Me ha encantado encontrar a alguien con tu forma de ver y entender la vida. A su media pipeta, Carlotita, por tantas risas y charleta. Al "exquisito" por tener siempre esas ganas y alegría en el cuerpo; y a todos aquellos que hacen que el labo sea un lugar, que aunque parezca que sólo se dice esto porque sea esta sección, para mí fantástico; Irene, Marta, Raúl, Raquel, Sandra, Jose Luis, Carolina, Macarena, Mati, Carlos, Adrián, Sebas, Esther, Sergio, Rosario, Elisa, Cristian … Además de todos aquellos que pasaron por el labo y con los que compartimos tiempo, aventuras y seguro que algún rotulador, pipeta, celo… como Cristina, Eva, Elsa, Elena, David y seguro que algunos más que me dejo. Dra López Parra compañera de conciertos y muchas risas en juegos de mesa, muchas suerte en vuestra aventura!

A la alegría del labo y estoy seguro que de allí a donde vayáis, Srta Picatoste y Sarita. No sabéis la felicidad que transmitís a todas y cada una de las personas a las que saludáis cada día y con las que compartís esas sonrisas. Estoy seguro que como todo el mundo tenéis vuestros días buenos y los menos buenos, pero hacéis que los menos buenos de los demás sean un poquito más felices sólo con vuestra presencia y eso es un don. Qué suerte tiene el Sr OL con vosotras, al que te deseo toda la suerte del mundo en esta etapa llena de ilusiones y desafíos como progenitor.

A toda la gente de la 4ª planta que siempre ha estado dispuesta a echar una mano o a compartir un simple saludo en el ascensor, por los pasillos o en el microondas, Bea, Cris, Mª Paz, Fer, Marta, Ana, Dani… A Flor que te vaya muy bien en tu nueva etapa, a Isabel siempre dispuesta a ayudar en lo que sea. A las chicas de oro (Beñat®) del animalario.

Y no me podía olvidar de Carmen, mira que es difícil conseguir que oiga tu "dulce" voz a 20 m, con tus "dulces" preguntas típicas sobre "¿Quién ha dejado esta caja ahí?", unidas a tus "dulces" capones en el cuello y aún así siempre consigas sacarme una sonrisa cada vez que la oigo. Cuánta compañía nos hemos hecho a altas horas y qué cuánto nos hemos reído, gracias Carmen!

Al 1.11, cuántos buenos ratos, risas, fiestas y si hubo algún mal momento, del que ya no tengo recuerdo casi, hemos conseguido convertirlo en risas! A la Pipe con su ímpetu en el trabajo, en la vida, eres una luchadora y una gran amiga, espero verte muy prontito de nuevo. A Vero, con sus risas, bailes y

tonterías incontables, qué bien lo pasé en el labo aunque a veces os vacile con lo del calendario… y junto con Ánder y Asier, mucha suerte en vuestra nueva etapa sois una familia maravillosa y cuidadme a la Vero que necesita muchos mimos ;) ! A la Mari, sabes perfectamente que las caquitas algún día dominarán el mundo, y éste será un lugar mejor. Ese dominio empezará de forma silenciosa, a través de acciones revolucionarias y poco vistas hasta ahora como la generosidad, la ayuda sin pedir nada a cambio, la bondad o la amistad y en ese momento estoy seguro que tú serás una mandamás de ese movimiento revolucionario. A la Dra Vanessa (con dos esses) sigue tan divertida, a Inma mucho ánimo en tu nueva etapa profesional y con tus peques, al resto del labo con la Dra Perona al frente por haberme dado la oportunidad de trabajar en su laboratorio. De manera especial quiero agradecer su esfuerzo a ISP por enseñarme a discernir sobre el bien y el mal, sobre lo que quiero ser como persona o como compañero de laboratorio ahora y en el futuro, millones de gracias porque esta lección fue dura pero ciertamente te esforzaste muchísimo en la misma y espero haber aprendido! A la Gallarda, Ascen y a todos los compis como Jorge, Diana o Jose que hicieron del IIB unos días fantásticos

Todo esto de aquí arriba sin unos amigos con los que reír, tomarse alguna cañuela o copa y salir alguna noche que otra, ¿qué sería?

A DHT, Alperto, Primo, Lotinen (hijo de famoso), Marañón, Tinto y Quili porque casi mis primeros 18 años de vida fuisteis vosotros y todavía lo seguís siendo. Creo que hay pocas cosas que me hagan sentir tan orgulloso como cuando nos presentamos y decimos que nos conocemos desde que teníamos 3 años, y que vamos ya para los 30!!!!

A los ferracinos por tantas juergas, risas, charlas, PPPs, a Valen, Alfonsiko, Carba, Villa, Migue, Correa y Txapitohhhh. A las niñas Ana, Pati, Mai e Inma… Sobretodo muchas gracias a los que estos útlimos 6 meses han tenido que aguantarme como compañero fantasma y sólo oyéndome hablar de esta tesis. Esta tesis empezó a gestarse, aunque de manera muy prematura, en el Aquinas con los batiditos "muy ricos" del Luigi, la pocha, novatadas, la típica mesa que cae del 6º piso de manera totalmente accidental… qué tiempos!

Y a todos aquellos que por falta de espacio no se pueden mencionar como la gente de la Uni (mal llamados guays), los del Maritornes (Campeón!!!)…

Finalmente no puedo dejar de agradecer a aquellos que han estado cerca de mí durante toda mi vida y sin cuyo apoyo, cariño, enseñanzas y sobretodo amor este trabajo hubiese sido imposible. Mis primos Miguel, Ángela (los pequeñuelos Alejandra y Juan, los más guapos de todo Logroño) y Mireia cuántos momentos para recordar en la Kawasaki con el tío Julio (no vamos a decir que infringiendo casi todas las normativas legales que ahora mismo, pero no entonces, están vigentes) en el chalet, pescando pulpos en Oyambre, saliendo por Logroño o de viajes varios por el mundo. Martita (te ha tocado el ita como a mí el ito pero al final te gustará y todo ya verás) mucha suerte en tu nueva aventura por mañolandia.

A mis tíos sin los cuales no entendería ni mi nacimiento (con Joaquín en primera línea) ni ningún otro momento de mi vida. Siempre habéis estado ahí y ojalá lo sigáis estando a cada paso que dé porque os necesito. Nunca acierto con el orden de edad pero espero medio guiarme; a Josechu y Nunchy por vuestra alegría de vivir, Julito y Ana por vuestra generosidad y bondad; Carmen y Helio por vuestro apoyo, conversaciones, libros, tortillas de patata y merlucita rebozada…Joaquín y Marta estuvisteis desde el primer día ahí y siempre estáis ahí para los demás; Mari Tere una madre en la sombra, siempre cuidando de los sobrinos, siempre echando una mano cuando has podido por todas partes, siempre con un comentario gracioso y agudo; a Pili y Rafa porque en la vida hay gente que crea, que da energía, que da ganas de vivir y esos sois vosotros, muchas gracias a todos por ser tan maravillosos!!! También quiero agradecer a otra parte muy importante de la familia como son la Julia y Narciso, a la primera por poner la semillita de la biología con la pirita, calcopirita, cuarzos y al segundo por ser el vínculo más cercano con la divinidad y con el que siempre hemos podido contar.

A los más cercanos, al Padre trabajador y luchador incansable por su familia. A mi padre en la sombra, sabes que siempre te he admirado y que he aprendido mucho de ti y espero seguir aprendiendo siempre. A mi madrina italiana, no sólo una hermana "encantadora" como diría ella misma seguida de una de sus sonrisas infinitas y preciosas, sino una madre coraje y con dos pares de *`´Ç.?. A Tt, la pequeñita sabes que siempre tendrás un huequito allí donde esté, bien 3 calles más allí o un pelín más lejitos! A mis cuñados, Patricia y Álvaro, y a mis sobris porque sois el futuro y espero que estéis orgullosos del mundo que os dejamos todos estos que aparecen a vuestro alrededor; Mencía, Teresa, Mateo, Mauro, Antia y Jimena.

A aquellos que no están, y me acuerdo especialmente de dos grandes personas con las que tuve la inmensa suerte de compartir gran parte de mi niñez, la abuela y Nati. Cuántas comidas en el Joto sin que supieran cómo estábamos relacionados, cuántos helados en la Tapiada saboreados en la siesta del chalet en verano, cuánto "Pente Tere pente" de camino por Barros. Cuánta sabiduría recogida tan sólo en dos

tomos, en 4 añejas piernas recogidas en el brasero de Calvo Sotelo, briscas, muses, los seises…. Abuela me sigue sin entrar en la cabeza cómo pudiste criar a 7 niños, en aquella época y que salieran todos tan bien, te confieso que me aterra pensar cómo puede ser criar si quiera a uno solo, y tú 7!!! Jamás te oí una mala palabra, te vi un mal gesto, te noté una mala mirada. Espero que te sientas al menos un poco orgullosa de todo lo que aprendimos de ti e intentamos reflejar cada día en nuestras vidas.

A la protagonista de esta tesis, siempre huyendo del foco pero esta vez no te puedes escapar porque tú diste lugar a todo esto. Tú estabas ese 28 de Diciembre a las 8 de la mañana en la Clínica Clavijo, como siempre te gusta recordar, nadie más, tú y yo, yo y tú desde ese día y cada día. Porque siempre me enseñaste a que el orgullo no sirve para nada, pero si algo me llena de orgullo es hablar de ti, porque no he conocido a nadie tan bondadoso como tú, por eso tú eres mi orgullo. Si sólo consiguiese ser una millonésima parte de lo bueno que tú eres y ser una millonésima parte del buen ejemplo que tú has sido para nosotros, sería aún así una gran persona. Porque tú mami te mercerías estar ahí arriba en el estrado contando a todo el mundo tu vida, porque eso si es una verdadera tesis, una tesis *Cum Laude* de verdad. Estoy seguro que si contases tu lucha, tu esfuerzo, tu inteligencia o tu amor sólo por unos segundos recibirías cualquier galardón que tenga un verdadera significado! Muchas muchas muchas muchas gracias MAMI porque todo esto es para ti!

Y por último a la persona que ha llegado de manera más reciente a esta familia tan numerosa, Carmen. Porque esta tesis te debe muchísimo a ti, quizás más que a mí, porque otro cualquiera habría podido hacer esta misma tesis pero yo no hubiera podido hacer esta tesis sin ti. Porque la vida está llena de casualidades, o no, y hay que hacer caso a las señales; un saxo hilvanando una conocida melodía de Frank Sinatra en Quebec "*I want to be a part of it*…", una ambulancia del Langome, un beso (o dos) en el Empire un 1 de Julio… Gracias por estar ahí siempre, en los buenos y en los malos momentos, en la ~~riqueza~~ y en la pobreza, porque cada día a tu lado sabes hacer que sea especial y eso hace que cada día a tu lado sea todavía si cabe mejor que el anterior. También quería mencionar a mi familia cántabra, Milagros, Carmen y Julián porque habéis sido y sois parte muy importante de este trabajo.

Gracias a todos porque sin todos y cada uno de vosotros esta tesis no hubiese sido posible y gracias también a los que hayan sido capaces de leerse las 6 páginas finales de Agradecimientos, y eso que inicialmente no quería escribir casi nada, que si era injusto, que si bla bla bla… si es que a uno se le va la fuerza por el teclado.

Resumen

La aterosclerosis es una enfermedad originada, entre otras causas, por la acumulación subendotelial de LDLs modificadas y en la que la inflamación y el estrés oxidativo juegan un papel fundamental. Las proteínas de respuesta al estrés (CSPs) son un grupo de proteínas que están presentes de manera ubicua y cuya síntesis se induce como mecanismo de protección frente a condiciones adversas, aunque algunas se expresan de manera constitutiva y son esenciales para la viabilidad celular. Numerosos estudios han demostrado que las CSPs están presentes en la placa de ateroma y pueden modular diversos procesos involucrados en la formación y desarrollo de la aterosclerosis, entre ellos la inflamación y el estrés oxidativo. En esta tesis estudiamos el papel en aterosclerosis de dos grupos de CSPs: las proteínas de respuesta al choque térmico (HSPs) y las proteínas de la familia de la Tiorredoxina (TRX). Estudios en placas ateroscleróticas humanas demuestran la presencia de HSP90 asociada principalmente a la zona inflamatoria y placas inestables. La inhibición de HSP90, tiene diversos efectos pleiotrópicos entre los que se encuentran la inducción de la respuesta a estrés y la modulación de numerosas rutas de señalización por degradación de las proteínas cliente de HSP90. Nuestros estudios en células implicadas en el proceso aterogénico [(monocitos/macrófagos y células del músculo liso vascular (CMLVs)] han demostrado que el uso de inhibidores de HSP90 modula la activación de numerosos mediadores de la respuesta inmuno-inflamatoria [p.ej JAK/STAT o el factor nuclear kappa B (NF-κB)] y oxidativa (MAPK o NADPH oxidasa). Para profundizar en estos resultados realizamos un modelo experimental de daño vascular [(aterosclerosis en ratones knock out para la Apolipoproteína E ($ApoE^{-/-}$)] en el que se obtuvieron resultados análogos a los anteriores, describiendo además una disminución del tamaño de la lesión y una reducción del contenido lipídico de las placas presentes en el arco aórtico. Otras CSPs como la Peroxirredoxina-1 (PRDX-1)/TRX también implicadas en aterosclerosis, pueden modular el estado redox celular en parte a través de su interacción con la NADPH oxidasa. Así, en estudios con monocitos/macrófagos describimos cómo el estrés oxidativo induce la liberación de PRDX-1/TRX en un proceso dependiente de la producción ión superóxido por la NADPH oxidasa. Además, mostramos que los niveles circulantes de PRDX-1/TRX están elevados en pacientes con aterosclerosis carotídea y se correlacionan con el espesor carotídeo de la íntima-media en estos pacientes, igualmente correlacionan con los niveles de superóxido dependiente de la NADPH oxidasa en pacientes asintomáticos.

Todos estos datos apoyan la utilidad potencial de las CSPs como posibles dianas diagnósticas y/o terapéuticas en enfermedades relacionadas con la inflamación y el estrés oxidativo, como la aterosclerosis.

Summary

Atherosclerosis can be defined as a chronic oxidative and inflammatory disease which originates from the subendothelial acummulation of modified LDLs. Cell stress proteins (CSPs) are a ubiquous group of proteins synthesized by cells as a defense mechanism against adverse conditions. Various studies have shown that CSPs are present in the atheroma plaque, and it was suggested that they can inhibit different processes involved in the origin and development of atherosclerosis, such as inflammation and oxidative stress. In this thesis we studied the role of two groups of CSPs; Heat shock proteins (HSPs) and the Thioredoxin (TRX) family, in the modulation of these processes in atherosclerosis. The inhibition of one of these HSPs, such as HSP90, has pleiotropic effects and among them are; increase of HSP70 levels and modulation of numerous signaling pathways due to the degradation of HSP90 client proteins [e.g. Janus Kinase (JAK)/STAT or MAPK)]. One of the numerous HSP90 client proteins is the catalytic subunit of the NADPH oxidase, NOX1, which requires HSP90 to fulfil its normal function. Thus, we found that the use of HSP90 inhibitors in cells involved in atherosclerosis [monocytes/macrophages and Vascular smooth muscle cells (VSMCs)] modulate inflammatory and oxidative responses. Besides, we found analogous results using an *in vivo* model of atherosclerosis [Apolipoprotein E KO mice ($ApoE^{-/-}$)], together with a decrease in total size lesion in the aortic root and a diminution in lipid content of the lesions. Another CSPs such as PRDX-1(Peroxiredoxin)/TRX are functionally related to NADPH oxidase. In studies with monocytes/macrophages we found that oxidative stress induce PRDX-1/TRX release, suggesting the potential involvement of NADPH oxidase dependent superoxide production and the increase of circulating PRDX-1/TRX levels in atherotrombosis. In addition, we show that circulating levels of PRDX-1/TRX are elevated in carotid atherosclerotic patients and correlate with the intima-media thickness and NADPH oxidase dependent superoxide production in asymptomatic subjects.

On the whole, theses data support the potential use of these CSPs as therapeutic targets in inflammatory and oxidative related diseases, such as atherosclerosis.

ÍNDICE

ABREVIATURAS

17-AAG	17-alilamino-17-demetoxigeldanamicina
17-DMAG	17-dimetilaminoetilamino-17-demetoxigeldanamicina
AAA	Aneurisma aórtico abdominal
Aha1	Activador-1 de la ATPasa de HSP90
$ApoE^{-/-}$	Ratón Knock out para la Apolipoproteína E
BAG-1	Antágeno asociado a BCL2
CCL	Quimioquina ligando tipo C-C
CD36	Clúster de diferenciación 36
CCR	Receptor de quimioquinas con motivo C-C
CHIP	Proteína que interacciona con HSP70/HSC70 por el extremo C terminal
CMLVs	Células del músculo liso vascular
CSPs	Proteínas de respuesta al estrés
DHE	Dihidroetidio
ROS	Especies reactivas de oxígeno
GSH	Glutation
GPx	Glutation peroxidasa
GR	Glutation reductasa
GSSG	Glutation oxidado
H_2O_2	Peróxido de hidrógeno
HO·	Radical hidroxilo
HO-1	Hemo-oxigenasa-1

HOP	Proteína organizadora de HSP70–HSP90
HSE	Elemento de choque térmico
HSFs	Factores de choque térmico
HSPs	Proteínas de choque térmico
IKK	Cinasa del inhibidor del factor nuclear kappa B alpha
LDLox	LDLs oxidadas
MCP-1	Proteína quimioatractante de monocitos-1 (también CCL-2)
MMP	Metaloproteinasa de matriz
MnSOD	Superóxido dismutasa del manganeso
NF-κB	Factor nuclear kappa B
NOXO1	Organizador 1 de NOX
O_2^-	Anión superóxido
PBMCs	Células mononucleares de sangre periférica
PMA	Forbol 12-miristato 13-acetato
PN	Red de proteostasis
PRDX	Peroxirredoxina
RANTES	Regulada bajo activación, secretada y expresada normalmente por células T (también CCL-5)
Th1	Linfocito cooperador tipo 1
TRX	Tiorredoxina
TRXR	Tiorredoxina reductasa
TXNIP	Proteína que interacciona con TRX [(también proteína de unión a la TRX (TRXBP) o proteína-1 sobre-expresada con vitamina D3 (VDUP-1)]

I. INTRODUCCIÓN

Las enfermedades cardiovasculares son la primera causa de muerte en el mundo, tanto en los países occidentales como en los países en vías de desarrollo [135]. De ellas, la aterosclerosis es la principal causa de esta morbimortalidad.

1.1. ATEROSCLEROSIS

La aterosclerosis es un proceso complejo que empieza a desarrollarse desde la juventud y progresa de manera asintomática hasta la edad adulta [248]. Implica múltiples procesos incluyendo disfunción endotelial, estrés oxidativo, inflamación, proliferación vascular, neovascularización, apoptosis, degradación de la matriz extracelular y trombosis [72]. Estudios recientes han resaltado la importancia de la inflamación y el estrés oxidativo en todos los estadíos de la aterosclerosis [6, 119, 131, 207].

La rotura de la placa y la oclusión del vaso son los dos procesos claves que dan lugar a eventos clínicos, como el infarto de miocardio o el ictus [4]. El conocimiento de la fisiopatología de la aterosclerosis y las enfermedades vasculares relacionadas ha cambiado a lo largo de la última década, proporcionando nuevas perspectivas para las estrategias de prevención y terapia.

1.1.1. Estructura de la pared vascular

La formación de la placa de ateroma en un proceso lento que puede iniciarse en edades tempranas y que afecta inicialmente a la capa más expuesta a la circulación, o íntima arterial, y secundariamente a la capa media. Las células que se encuentran en mayor medida en la lesión ateromatosa son: leucocitos (monocitos que pueden diferenciarse a macrófagos, neutrófilos y linfocitos T), células del músculo liso vascular (CMLVs), además de eritrocitos y plaquetas [231-233].

Las arterias sanas están formadas por tres capas (Figura 1):

- *Capa íntima*: Compuesta por una capa de células endoteliales que reviste la superficie interna del vaso y una membrana limitante elástica interna, que la separa de la media. El endotelio se apoya en la capa subendotelial, constituida por tejido conjuntivo que puede presentar CMLVs de manera dispersa. La membrana elástica es tubular y perforada, permitiendo la difusión de los metabolitos que nutren las células de la pared arterial.
- *Capa media*: Formada principalmente por CMLVs dispuestas circularmente a las que se agregan cantidades variables de elastina, colágeno y proteoglicanos. Las CMLVs son células metabólicamente muy activas y responsables de la síntesis de la matriz extracelular. La media posee una lámina elástica externa que la separa de la capa adventicia.
- *Capa adventicia*: Consta principalmente de fibras de colágeno y elastina. Esta capa continúa con el tejido conjuntivo y adiposo que envuelve los *vasa vasorum*, los cuales desempeñan una función principalmente estructural y nutritiva.

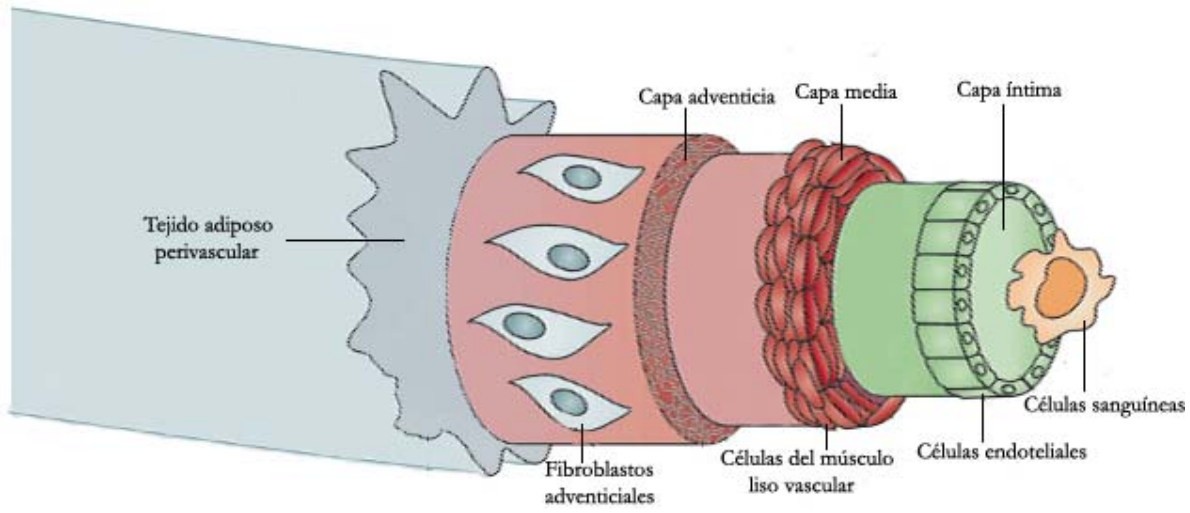


Figura 1. Estructura de la pared vascular en un vaso sano (modificado de Drummond* et al *[49]).

1.1.2. Patogenia de la aterosclerosis

En la aterosclerosis se pueden distinguir varias fases. En la fase temprana los niveles circulantes elevados de LDLs inducen su acumulación intimal y su extravasación a la pared vascular, donde se acumulan y son oxidadas (LDLox). Las LDLs, y en particular las LDLs modificadas, participan en todos los pasos de la aterogénesis, siendo la oxidación de las LDLs una de las modificaciones más relevantes en la aterosclerosis, de hecho la hipercolesterolemia es el mayor factor de riesgo para el desarrollo de la enfermedad ateromatosa. En humanos, mutaciones en el receptor de las LDLs (hipercolesterolemia familiar) conlleva un fuerte incremento en la concentración plasmática de LDLs, favoreciendo el desarrollo de la placa ateromatosa y complicaciones asociadas que pueden dar lugar a eventos cardiovasculares a partir de los 30 años (infarto de miocardio, accidentes cerebrovasculares, etc...). Estas LDLs oxidadas inducen la expresión de moléculas de adhesión y la secreción de factores quimiotácticos, incrementando la adhesión y migración de los leucocitos (monocitos, linfocitos y neutrófilos). Una vez dentro de la íntima, los monocitos se diferencian a macrófagos y junto con las CMLVs se van cargando de LDLs modificadas a través de los receptores basurero como el clúster de diferenciación 36 (CD36) [53], formándose las células espumosas e iniciando la activación de las CMLVs [194]. La acumulación neointimal de las células espumosas contribuye de manera fundamental al desarrollo e inestabilidad de la placa. La migración y el aumento en el número de células espumosas depende del receptor CD36 y está mediada por la producción de especies reactivas de oxígeno (ROS) fundamentalmente generadas por la actividad enzimática de la NADPH oxidasa [176] (Figura 2). La íntima representa un espacio muy limitado en las arterias sanas donde la acumulación de células fagocíticas, o células espumosas debido a su aspecto vacuolado, ha sido detectada en arterias fetales humanas, en particular en casos de hipercolesterolemia

materna [172]. El aumento en el número de células espumosas da lugar a las estrías grasas. Por su parte, las CMLVs producen mediadores pro-inflamatorios como la proteína quimiotáctica de monocitos [MCP-1, (también quimioquina ligando-2 tipo C-C, CCL-2)] y la molécula inductora de adhesión celular-1 [216]. La evolución de las estrías grasas hacia el ateroma fibroso se promueve por la proliferación de CMLVs dentro de la íntima que forma la capa fibrosa rodeando las células espumosas y lípidos extracelulares acumulados (núcleo lipídico), caracterizado por un cambio en las CMLVs de un fenotipo contráctil a otro secretor. Las placas fibro-ateromatosas evolucionan hacia lesiones más complicadas y heterogéneas pero frecuentemente caracterizadas por la presencia de material esclerótico (calcificaciones) y la formación de un núcleo necrótico, lipídico y hemorrágico compuesto por debris celular, células sanguíneas e inflamatorias (leucocitos, plaquetas y eritrocitos). Conjuntamente, en ambientes inflamatorios y oxidativos la pérdida de CMLVs, posiblemente por apoptosis, junto con el aumento en la expresión de enzimas proteolíticas, conducen a la reducción del contenido de colágeno de la placa, favoreciendo su inestabilidad y rotura final [131, 143, 217]. Los vasos normales se nutren a través de la difusión de oxígeno del lumen de los vasos o del vasa vasorum adventicial. Cuando la pared del vaso excede la distancia efectiva de difusión del oxígeno, los *vasa vasorum* proliferan en las capas internas de la pared del vaso, donde normalmente estaría ausente. Los macrófagos atraídos y cargados de LDLs son uno de los responsables de la producción de citoquinas que estimulan el crecimiento de neovasos [182]. La neoangiogénesis y la hemorragia intra-placa dirigen a la lesión a todos los componentes sanguíneos, incluyendo eritrocitos, leucocitos, plaquetas y proteínas plasmáticas. La presencia de hemorragia dentro de la placa ha sido descrita como uno de los mayores determinantes de eventos clínicos en pacientes con enfermedad carotídea [76]. La sangre de la placa refleja una hemorragia local y está asociada a un incremento en la formación de neovasos. La sangre provee a la placa de un ambiente prooxidativo, pro-inflamatorio y proteolítico que puede promover la vulnerabilidad de la capa fibrosa, induciendo la apoptosis de células vasculares, incluidas las CMLVs. La desestabilización, rotura y consiguiente formación de un trombo, es la base de las consecuencias clínicas más severas de la aterosclerosis (Figura 2).

Diversos **modelos experimentales** se han utilizado ampliamente para profundizar en la patogenia del proceso aterosclerótico, entre ellos, los ratones deficientes o mutados en el receptor de las LDLs se usan de manera habitual como modelos de aterosclerosis, así como ratones deficientes para la apolipoproteína E ($ApoE^{-/-}$) o conejos Watanabe con hiperlipidemia hereditaria. El ratón knock out para ApoE fue creado mediante inactivación genética dirigida dando lugar a un cepa que desarrolla de manera espontánea aterosclerosis [179]. Alimentado con una dieta estándar los $ApoE^{-/-}$ muestran unos niveles de colesterol de 500 mg/dL, la mayor parte

como lipoproteínas de muy baja densidad y fracciones remanentes de quilomicrones. Con una dieta tipo "occidental" (rica en grasas) estos niveles se multiplican por 2-3 veces [180]. El ratón ApoE$^{-/-}$ ha sido el modelo elegido en esta tesis ya que las lesiones que desarrolla contienen el espectro entero de lesiones observadas en aterogénesis [280, 180]. De hecho, un análisis cronológico de los eventos secuenciales implicados en el desarrollo de la lesión muestra que son particularmente similares al desarrollo en humanos. Los sitios que con mayor predilección por la formación de placa son por este orden: el arco aórtico, el tronco braquiocefálico, la carótida izquierda y las arterias sub-clavia y coronaria [20, 88]. El modelo de ApoE$^{-/-}$ es una herramienta útil para: (i) identificar susceptibilidades en aterosclerosis mediante la modificación genética, mediante los métodos del gen candidato y el mapeo genético, (ii) descifrar los mecanismos moleculares y los tipos celulares implicados en aterosclerosis, (iii) estudiar los efectos de fármacos en aterosclerosis, y (iv) el análisisis de nuevas terapias que prevengan la progresión de la lesión [277].

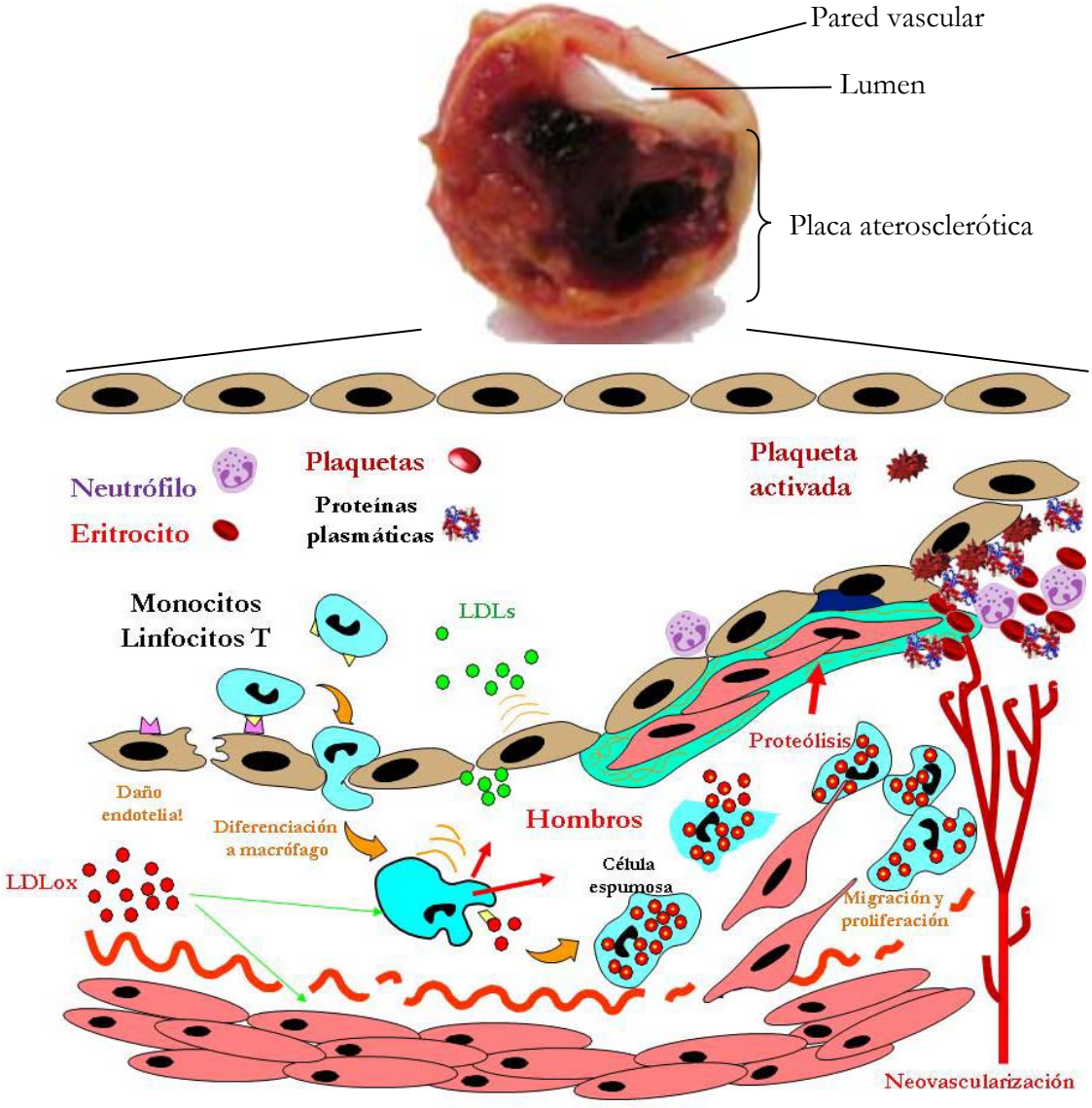


Figura 2. Vista macroscópica de una placa aterosclerótica con hemorragia interna (modificada de Michel* et al *[151]) y esquema del desarrollo de la lesión aterosclerótica.

1.1.3. Balance redox en aterosclerosis

La aterosclerosis es una enfermedad inmuno-inflamatoria crónica, originada por la acumulación subendotelial de LDLs, que pueden ser oxidadas por ROS. Pero el estrés oxidativo no sólo está envuelto en los estadíos iniciales (modificación de LDLs o NO), sino también en estadíos más avanzados, como por ejemplo en la estimulación de proteasas que contribuyen al debilitamiento de la capa fibrosa del ateroma [74, 138]. Así, dos procesos fundamentales, como son la inflamación y el estrés oxidativo, mediados por ROS [6, 119] interactúan para promover y agravar el fenómeno aterosclerótico. Por otro lado, las ROS tienen diferentes acciones en la vasculatura como la modulación de vías de señalización a través de cambios en la actividad de cinasas sensibles al estado redox (por ej. las MAPK), factores de transcripción [por ej. el factor nuclear kappa B (NF-κB)] u otras moléculas [por ej. Tiorredoxina (TRX)] [51]. Hay diferentes tipos de ROS como el NO, anión superóxido ($O_2^{\cdot}$), el peróxido de hidrógeno (H_2O_2) y el radical hidroxilo (HO·). El NO es normalmente producido por las sintasas del NO en el endotelio vascular mientras que el superóxido se genera mediante la reducción de un electrón de oxígeno a través de varias formas de oxidasas como la xantina oxidasa, la NADPH oxidasa, la citocromo P450 mono-oxigenasa mitocondrial, la hemo-oxigenasa y la lipo-oxigenasa. Por su parte, el peroxinitrito es rápidamente generado mediante la reacción de NO con superóxido [67].

Los altos niveles de ROS pueden producir daños directos a las macromoléculas, como lípidos, ácidos nucleicos y proteínas [26]. Debido a la presencia de estructuras bis-alílicas en los ácidos grasos poli-insaturados, los lípidos son una de las dianas más sensibles a la oxidación por ROS. Una vez que se inicia la peroxidación de lípidos, se da lugar una propagación de reacciones en cadena que sólo terminarán una vez se haya producido el producto final. De esta forma, productos finales de la peroxidación lipídica como la LDL modificada malondialdehído [81] se acumulan en la circulación. Las bases de DNA son, igualmente, muy susceptibles a la oxidación por ROS, y el producto predominante que permite detectar la oxidación de las bases de DNA *in vivo* es la 8-hidroxi-2-deoxiguanosina. La oxidación del DNA puede causar mutaciones y deleciones tanto en el DNA nuclear como en el mitocondrial. En concreto, el DNA mitocondrial es especialmente sensible al daño oxidativo debido tanto a su proximidad a un sitio primario de producción de ROS, como a su deficiente capacidad de reparación en comparación con el DNA nuclear. Casi todos los residuos de aminoácidos que forman parte de una proteína pueden ser oxidados por ROS. Algunas formas de aminoácidos oxidados muy estudiadas incluyen la formación de puentes disulfuro en los residuos de cisteína, derivados carbonilados o residuos de metionina-sulfóxido. Estas modificaciones oxidativas pueden llevar a cambios funcionales en numerosos tipos proteicos, lo que puede dar lugar a un impacto fisiológico considerable. Por

ejemplo, el daño oxidativo en las enzimas puede causar una modificación en su actividad, mientras que el daño en proteínas estructurales y chaperonas puede producir agregación proteica. Igualmente, la modulación redox de los factores de transcripción, puede producir cambios en su actividad de unión al DNA, lo que finalmente da lugar a cambios en la expresión génica que afectan a procesos asociados con la aterosclerosis [113].

Diversos factores, como el forbol-12-miristato-13-acetato (PMA) [59] o el TNF-α [57, 116], pueden regular la producción de ROS. El PMA activa la producción de ROS a través de la cinasa de la proteína C [59], un mecanismo común subyacente en procesos hemodinámicos [246] y humorales [89]. Además, el PMA estimula la diferenciación de monocito a macrófago, un proceso clave en el desarrollo de la lesión ateromatosa y que está íntimamente relacionado con el estrés oxidativo [235]. Por su parte, el TNF-α no sólo es capaz de estimular la producción de ROS [129], sino de activar diversas MAPK implicadas en estrés oxidativo, como por ejemplo ERK1/2 [82]. ERK1/2 ha sido ampliamente descrito en aterosclerosis ya que es un mediador de la señalización por IFN-γ en macrófagos humanos en los que controla la activación de STAT1 y la incorporación de LDLs modificadas [128]. Además modula la síntesis de la metaloproteinasa de matriz-9 (MMP-9), una de las principales MMPs responsable de la degradación de proteínas de matriz extracelular [109]. Por otro lado, ERK1/2 está implicado de manera clave en el estrés oxidativo presente en la lesión vascular regulando la producción de ROS dependiente de la NADPH oxidasa [156]. Entre los sistemas productores de ROS, el sistema oxidasa de la NADPH es uno de los principales dentro de la pared vascular, y está presente en células endoteliales, CMLVs, fibroblastos y monocitos/macrófagos [116]. Numerosos estudios han demostrado el papel fundamental de las NADPH oxidasas vasculares y fagocíticas en el desarrollo de las enfermedades vasculares en humanos [116]. De hecho, se ha demostrado recientemente que la actividad NADPH oxidasa correlaciona positivamente con el espesor carotídeo de la íntima-media en sujetos asintomáticos [275]. Además, una reducción de la actividad de NADPH mediante el uso de un inhibidor de la ruta de JAK/STAT está asociada con una disminución en el tamaño de la lesión aterosclerótica [57].

Las NADPH oxidasas son multi-enzimas asociadas a la membrana celular cuya estructura clásica en fagocitos está compuesta por cinco subunidades: tres citosólicas (p67phox, p47phox y p40phox); y dos asociadas a membrana (gp91phox y p22phox) que forman parte del citocromo b588 [14]. Se han descrito numerosos homólogos del núcleo catalítico gp91phox (NOX2) en células no fagocíticas, incluidas las vasculares [3, 141], que constituyen la familia de las proteínas NOX formada por 7 miembros (NOX1-5 y DUOX1-2). En los grandes vasos, las NADPH oxidasas predominantes de las CMLVs son NOX1 [117] y NOX4 [52, 80] y ambas contribuyen a la producción de ROS. NOX4 es una enzima constitutiva con baja actividad catalítica que se relaciona con

procesos de señalización fisiológica. En cambio, NOX1 es inactiva en condiciones basales [63] y tiene bajos niveles de expresión, sin embargo muestra una alta actividad catalítica y un aumento en sus niveles bajo condiciones patológicas, estando asociada al daño cardiovascular [47, 48]. En CMLVs, NOX1 forma un complejo con p47phox y su homólogo el organizador-1 de NOX (NOXO1). NOXO1 está constitutivamente activo porque carece de un factor inhibitorio, que sí posee p47phox [38], siendo NOXO1 necesaria para la producción de altos niveles de ROS a través de NOX1 [240]. Además, NOXO1 está unido a la membrana de células no estimuladas, colocalizando con NOX1 y manteniendo su actividad catalítica [38]. Además, NOX1 como proteína cliente (aquella proteína que necesita de la interacción con una chaperona molecular, de forma transitoria, para conseguir su correcta conformación final) de la proteína de choque térmico (HSP)90, requiere también de su interacción para la estabilidad de la enzima y producción de ROS [36].

Sin embargo, el balance redox es sólo posible gracias a la acción coordinada de los sistemas pro-oxidantes y de la maquinaria antioxidante celular, principalmente el sistema de la TRX (apartado 1.2.2.), el glutation (GSH) o la enzima superóxido dismutasa del manganeso (MnSOD). El GSH forma parte del principal sistema que se encarga del mantenimiento y regulación de estado tiol-redox celular [148]. El estado redox de la pareja GSH reducido/GSH oxidado (GSSG) es un indicador clave del ambiente oxidativo [102], por lo que cambios en el ratio de esta pareja modulan numerosos procesos implicados en aterosclerosis [108]. En su estado reducido, el GSH protege a las células frente al daño producido por el estrés oxidativo gracias a su capacidad de eliminar los peróxidos lipídicos [148]. La síntesis de GSH se produce intracelularmente, desde donde se dirige a los diferentes compartimentos celulares, mientras que su degradación ocurre en el espacio extracelular, por lo que su transporte es clave para el proceso de reciclaje del GSH [17]. Sin embargo, la vida media en el plasma sanguíneo es extremadamente corta, desde segundos a unos pocos minutos [148]. Dada la importancia de los procesos regulados por los niveles de GSH, no es extraño que las alteraciones en sus niveles se hayan relacionado con numerosas enfermedades [60]. Por otro lado, la MnSOD es una enzima mitocondrial que tiene un papel fundamental en la protección, frente al estrés oxidativo producido por los radicales superóxido, en la mayoría de células de mamíferos, incluidas las de origen cardiovascular [61]. La MnSOD funciona como una defensa esencial antioxidante, mediante la protección de dianas críticas del superóxido [61], además de prevenir la formación de radicales libres más tóxicos.

Así, debido a que el estrés oxidativo está envuelto en el origen y desarrollo de la lesión aterosclerótica, existe un interés creciente en el desarrollo de nuevas terapias que prevengan el aumento patológico de ROS en la pared vascular [127].

1.1.4. Respuesta inmuno-inflamatoria en aterosclerosis

La inflamación tiene un papel clave en el desarrollo de la lesión aterosclerótica [72, 131]. La respuesta inflamatoria altera el comportamiento fisiológico de las células de la pared vascular e induce el reclutamiento de nuevas células inflamatorias que favorecen la formación de la lesión y futuras complicaciones clínicas. Se ha descrito que las lesiones responsables de los eventos agudos no tienen que ser críticamente obstructivas [249]. Hay dos causas principales que originan eventos clínicos: la erosión endotelial sin rotura del núcleo lipídico y la fractura de la placa en la zona fibrosa que rodea al material lipídico [56]. Las placas que sufren erosión son ricas en CMLVs y proteoglicanos en la superficie luminal y suelen ser menos calcificadas que las que sufren rotura. La rotura de la placa es detectable en aproximadamente el 60-70 % de los casos [55], ya que expone al torrente sanguíneo el material protrombótico del núcleo de la placa formado por fosfolípidos, factores tisulares y/o plaquetarios. La rotura de la placa ocurre preferentemente en las zonas en las cuales la placa es delgada o está parcialmente destruida por lo que el grosor de la capa fibrosa es un determinante clave de la estabilidad de la placa [198]. La capa fibrosa confiere resistencia a la rotura debido a su composición en colágeno y otras proteínas de la matriz extracelular sintetizadas por las células vasculares. La zona proclive a la rotura se denomina "hombro" de la lesión y se define como la parte de la placa que se encuentra a ambos lados del core lipídico y la cápsula fibrosa y que está en el borde del ateroma. En la región de los hombros la infiltración de macrófagos es muy superior respecto a la de la cápsula mientras que el número de CMLVs muestra un patrón opuesto [98, 142]. En las zonas de rotura se encuentran además células inmuno-inflamatorias (macrófagos y linfocitos T) activadas que son capaces de liberar moléculas pro-inflamatorias y diferentes proteasas (estas también son liberadas por las CMLVs activadas con fenotipo secretor), induciendo la activación de células que se encuentran en el core de la placa y la degradación de las proteínas de matriz extracelular, promoviendo de esta forma la inestabilidad y rotura de la placa [30].

Factores de transcripción implicados en la respuesta inmuno-inflamatoria

Las respuestas celulares están mediadas por proteínas cuya expresión génica se controla por diversos factores de transcripción. Un factor de transcripción es una proteína que se une a secuencias específicas de DNA controlando de esta forma el flujo de información genética desde el DNA al RNAm[118]. En general, los genes regulados por estos factores de transcripción permanecen en estado silente o tienen una mínima actividad, regulando así los procesos biológicos y fisiológicos. Sin embargo, en ciertas condiciones, la activación de estos genes se desencadena bruscamente o de forma crónica mediante un mecanismo controlado, al menos, en

parte por factores de transcripción. Dos factores implicados en la respuesta inmuno-inflamatoria en aterosclerosis como son JAK/STAT o NF-κB han sido estudiados en esta tesis.

La vía de JAK/STAT es una importante ruta de señalización asociada a los receptores de diversas citoquinas pro-inflamatorias, además también puede ser activada por insulina o IgGs [1]. La respuesta de las células a las citoquinas implica la unión del ligando a su receptor celular y su posterior oligomerización, produciendo los cambios conformacionales que inducen la activación de JAK asociada al receptor. JAK fosforila las tirosinas citoplasmáticas del receptor, creando sitios de reconocimiento SH2 (dominio de homología-2 Src) y este nuevo estado del receptor es reconocido por las proteínas STAT del citoplasma que son también fosforiladas por JAK. Entonces, las proteínas STAT activadas dimerizan y se translocan al núcleo donde activan la transcripción de distintos genes pro-inflamatorios [267].

Por su parte, NF-κB ha sido descrito en diversos tipos celulares y puede ser activado por un gran número de estímulos fisiológicos y no fisiológicos como citoquinas, mitógenos, virus, estrés mecánico u oxidativo y agentes químicos [68]. Las subunidades que constituyen NF-κB forman una familia de proteínas que comparten una región central muy conservada, el dominio de homología Rel [122], implicado en la unión al DNA, en la interacción con la subunidad inhibitoria IκBα y en la dimerización. Este dominio contiene además la señal de localización nuclear que facilita la translocación al núcleo. Aunque se han detectado varias formas diméricas del NF-κB, la clásica es el heterodímero constituido por las subunidades p50 y p65, que contiene los dominios de transactivación necesarios para la inducción de la expresión de genes [15]. La activación de NF-κB requiere la fosforilación de IκBα por la cinasa del inhibidor del factor nuclear kappa b alpha (IKK) y la degradación de IκBα por el proteosoma, quedando libre el dímero p50/p65 y permitiendo su translocación al núcleo. Al entrar al núcleo se unirá a secuencias específicas regulando la transcripción de genes clave en inflamación, proliferación o apoptosis [18].

Citoquinas implicadas en la respuesta inmuno-inflamatoria

Las citoquinas son moléculas de señalización secretadas de manera ubicua por las células. Funcionan como comunicadores intercelulares y se pueden clasificar de manera general en interleuquinas y quimioquinas. Las interleuquinas se denominaron así inicialmente debido a que su diana presumible eran los leucocitos, sin embargo en la actualidad incluyen un gran y diverso grupo de citoquinas. Por su parte las quimioquinas se llaman así por ser mediadores de la quimiotaxis celular. Tanto la vía de señalización JAK/STAT como NF-κB son activadas por citoquinas pro-aterogénicas y controlan la expresión de diferentes genes pro-inflamatorios, como IL-6, MCP-1, o la regulada bajo activación, secretada y expresada normalmente por células T

(RANTES, o también conocida como CCL-5). De hecho, las terapias experimentales que modulan la activación tanto de NF-ϰB como de STAT disminuyen los procesos inflamatorios en modelos de aterosclerosis [136, 170]. IL-6 contribuye tanto al desarrollo de la placa como a la desestabilización a través de diferentes mecanismos. Estos incluyen la liberación de otras citoquinas pro-inflamatorias, la oxidación de LDLs mediante fosfolipasas, la secreción de proteínas de fase aguda, la liberación de mediadores protrombóticos o la activación MMPs. Además, la formación de ROS por los sistemas enzimáticos vasculares puede jugar un papel clave en la regulación de IL-6, indicando una retroalimentación entre la oxidación y la respuesta inflamatoria [214]. Por su parte, tanto MCP-1 como su receptor, el receptor 2 de quimioquinas con motivo C-C (CCR-2), son muy importantes en el desarrollo de la respuesta inflamatoria y son cruciales en el reclutamiento de células inmunes, como los monocitos, hacia el interior de la lesión aterosclerótica [126, 274]. Por otro lado, MCP-1 juega un papel principal en el desarrollo de la aterosclerosis ya que el bloqueo de la vía MCP-1/CCR-2 resulta en la disminución del tamaño de la placa mediante la inhibición de la adhesión de monocitos a la pared vascular y la reducción del contenido en macrófagos en la lesión [94, 165]. Finalmente, el papel tanto de RANTES como de sus receptores CCR-1 y CCR-5 han sido profundamente estudiados en aterosclerosis, describiéndose por ejemplo, que RANTES puede ser transportada por plaquetas activadas dando lugar al reclutamiento de monocitos a la lesión aterosclerótica [91, 250].

La existencia de un componente inflamatorio en el desarrollo de la lesión vascular ofrece nuevas oportunidades para la prevención y tratamiento de las enfermedades cardiovasculares. A este respecto, se ha demostrado que terapias tanto clínicas como experimentales que disminuyan la inflamación en aterosclerosis pueden tener efectos beneficiosos mediante la prevención de la progresión de la enfermedad [24, 136, 170].

1.2. PROTEÍNAS DE RESPUESTA AL ESTRÉS

Las proteínas de respuesta al estrés (CSPs) han sido generalmente conocidas como HSPs, debido al descubrimiento de las HSPs en las glándulas salivares de *Drosophila melanogaster* como respuesta a un choque térmico [205, 242]. Sin embargo, existe un enorme rango de diferentes tipos de estrés que inducen la expresión de estas proteínas, tales como infección vírica, aumento de citoquinas, estrés oxidativo, ausencia de glucosa, o exposición a toxinas y ciertos metales pesados [11]. Por lo que el término de CSPs sería más ajustado en la actualidad. La relación entre la inducción en la transcripción de genes de HSPs, el papel en el ensamblaje de proteínas en respuesta al estrés y su papel como chaperonas fue descrito en profundidad hace varias décadas [8, 77, 243]. Así, al final de la década de los 80, se describieron numerosas proteínas celulares requieren

ayuda para su ensamblaje y que es facilitada por la acción de la familia de proteínas denominada "chaperonas moleculares" [77]. La definición más aceptada del término chaperona molecular es que son un gran grupo, y diverso, de proteínas que comparten la propiedad de asistir las uniones/desuniones no covalentes de otras estructuras macromoleculares, pero que no son componentes permanentes de estas estructuras cuando estas están cumpliendo sus funciones biológicas normales. Dentro de este heterogéneo y amplio grupo de chaperonas moleculares se encuentran entre otras, dos familias de proteínas implicadas en los procesos ateroscleróticos como son las HSPs y las proteínas de la familia de la TRX.

1.2.1. Proteínas de choque térmico

Las HSPs se clasifican de acuerdo a su peso molecular, variando desde 10 a 110 kDa [107]. Pertenecen a una familia de proteínas altamente conservada que expresan todas las células y organismos, desde las bacterias a los humanos, en respuesta al estrés, incluyendo como tal la exposición a metales pesados, citoquinas pro-inflamatorias, análogos de aminoácidos, estrés oxidativo o isquemia [37]. La expresión de las HSPs puede ser constitutiva o inducible. Se las considera generalmente como moléculas protectoras frente a diferentes tipos de estrés y tienen numerosas funciones dentro de la célula como chaperonas, asegurando el correcto ensamblaje de las proteínas recién sintetizadas o desnaturalizadas [196], inhibiendo la apoptosis [175] o manteniendo la integridad celular mediante la estabilización del citoesqueleto [158]. Las HSPs tienen diversas funciones extracelulares y los mecanismos a través de los cuales estas HSPs salen de las células están empezando a ser descritos. Algunas HSPs como la HSP90 muestran altos niveles incluso en células no estresadas, mientras que otras como HSP70 poseen una forma constitutiva y otra inducible que se activa por estrés [157, 101]. Así el papel de las HSPs, tanto en células normales como en aquellas sometidas a estrés, requiere la existencia de un proceso regulador complejo que asegure el correcto patrón de expresión de estas proteínas. De hecho, estos procesos deben estar operativos desde las etapas más tempranas del desarrollo embrionario, ya que los genes que codifican para HSP70 y HSP90 están entre los primeros genes embrionarios en ser expresados [163, 251]. La inducción de HSPs en respuesta a diferentes tipos de estrés depende de la activación de unos miembros específicos de una familia de factores de transcripción, los factores de choque térmico (HSFs), los cuales se unen a la secuencia consenso del elemento de choque térmico (HSE) en los promotores que codifican para genes de HSPs [5] (Figura 3). Se han clonado y caracterizado funcionalmente cuatro HSFs (1-4) en diferentes organismos. Sólo HSF1 y 3 están implicados en la regulación de HSPs en respuesta al estrés térmico, mientras que HSF2 y 4 participan en la regulación de HSPs en células no estresadas, siendo sus niveles regulados en

respuesta a un amplio rango de procesos biológicos, como la activación inmune y la diferenciación celular [5].

La respuesta al choque térmico se dispara por una gran variedad de tipos de estrés que interfieren con el correcto ensamblaje de las proteínas, dando lugar a la acumulación de proteínas mal ensambladas o agregadas. La respuesta al choque térmico es mediada por el HSF1 al unirse al HSE, presente en la región del promotor de un gran número de genes diana, incluidas las HSPs [9] (Figura 3). Bajo condiciones basales, HSP70 [221] y HSP90 [7] se mantienen unidas al HSF1 monomérico y a otras chaperonas en el compartimento citoplasmático [por ej. la proteína organizadora de HSP70–HSP90 (HOP) [226], o la proteína que interacciona con HSP70/HSC70 por el extremo C-terminal (CHIP) [83], miembros de la familia de la HSP40/DnaJ [152] o p23 [92]]. Bajo condiciones de estrés, HSF1 es liberado, se transloca al núcleo, trimeriza y activa la síntesis de HSPs [16, 211, 257] (Figura 3). De hecho, un mecanismo de retroalimentación negativo regula la respuesta al estrés, ya que los altos niveles de HSPs secuestran el HSF1 citosólico e impiden, por lo tanto, su translocación al núcleo y la consiguiente síntesis de HSPs (Figura 3). Las complejas interacciones entre chaperonas, cochaperonas y sus proteínas cliente deciden el destino de las proteínas desensambladas: bien un nuevo intento de ensamblaje o bien la ubiquitinación y posterior degradación mediante la vía del proteosoma. Sin embargo, en condiciones de estrés oxidativo extremo, la ubiquitinación puede ser sorteada y la degradación proteica se realiza de manera más directa, pero menos controlada, para evitar la acumulación excesiva de proteínas dañadas [223].

La demostración de que las HSPs juegan un papel muy importante en procesos relacionados con la evolución de la aterosclerosis está siendo cada vez más notoria, por lo que su estudio individualizado es fundamental para entender su papel fisiopatológico.

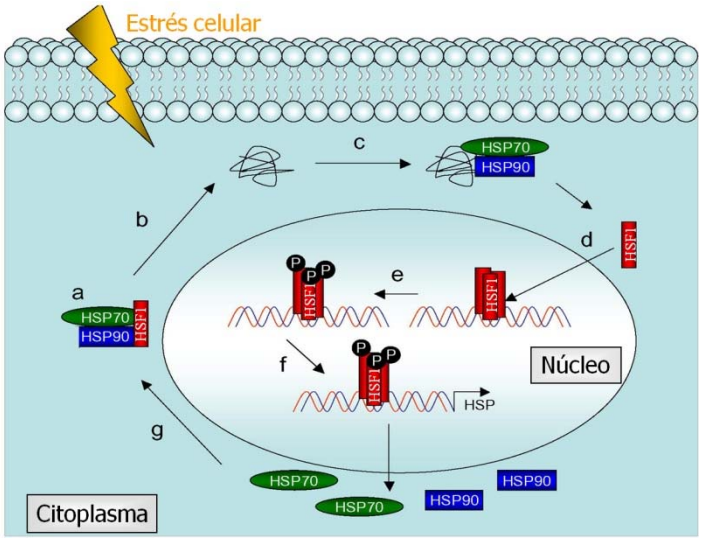


Figura 3. Esquema de la respuesta al choque térmico (modificado de Powers* et al *[186]). *(a) Bajo condiciones normales HSF1 se encuentra como monómero en estado reprimido por interacción con HSP90 y HSP70. (b) El estrés celular causa un incremento en proteínas desnaturalizadas (c) lo que lleva a la disociación de HSP90 y HSP70 de HSF1. (d) HSF1 monomérico se encuentra libre para trimerizar, translocar al núcleo y (e) sufrir una serie de fosforilaciones post-transcripcionales. (f) HSF1 activa la transcripción de numerosos genes de HSPs,incluyendo HSP90 y HSP70. (g) El incremento en la concentración celular de estas chaperonas lleva a la inactivación de HSF1 por la unión a la forma monomérica o trimérica de HSF1 [186].*

1.2.1.1. HSP90

HSP90 es una chaperona molecular ubicua y muy abundante, ya que sus niveles oscilan entre el 1 y el 2% del total de la proteína de la célula bajo condiciones normales. Está implicada en el ensamblaje, maduración y activación de numerosas proteínas, incluyendo algunos mediadores clave de la transducción de señales y la regulación transcripcional [191]. Funciona como parte de un complejo multi-chaperona a través de la asociación con cochaperonas (por ej. HSP70) y numerosas proteínas clientes (IKK, STAT, MEK o NOX1). La base molecular de la especificidad de las proteínas cliente por HSP90 se desconoce por el momento, ya que proteínas muy similares muestran diferente dependencia de HSP90 para su correcta funcionalidad. Cuando una proteína está excesivamente dañada o debido al uso de inhibidores de HSP90 (Figura 4), la proteína cliente es liberada y ubiquitinada por una ligasa E3 y degradada por el proteosoma [46]. La principal cochaperona de HSP90 es HSP70 [111, 269], aunque también se ha descrito un mecanismo de ubiquitinación dependiente de CHIP. Sin embargo el mecanismo de ubiquitinación dependiente de CHIP todavía no está claro. En ambos casos, HSP90 ayudado por HSP70 o por CHIP media en el proceso de "control de calidad" de las proteínas, mediante la monitorización de posibles objetivos para la degradación.

Debido a su papel como chaperona estabilizadora de muchas proteínas oncogénicas, se han llevado a cabo considerables esfuerzos para el desarrollo de compuestos terapéuticos que **inhiban HSP90**. Dentro del enorme grupo de compuestos que inhiben la actividad de HSP90, se encuentran un grupo de productos naturales como son las benzoquinonas ansamicinas. Uno de los primeros derivados de las ansamicinas estudiados fue la geldanamicina (Figura 4).

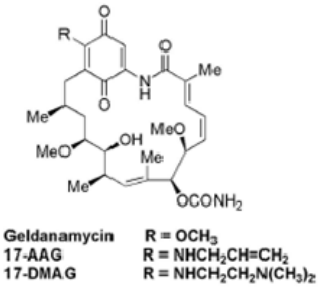


Figura 4. Estructura química de la geldanamicina y derivados (tomado de Powers* et al *[186]).

La geldanamicina y sus derivados compiten con el ATP por el sitio de unión a éste en HSP90. El ATP es necesario para la función chaperona de HSP90 [258]. A pesar de unos prometedores resultados en la actividad antitumoral tanto *in vitro* como *in vivo*, la geldanamicina resultó tener demasiados efectos secundarios adversos para su uso clínico [237]. Nuevos análogos de la geldanamicina se han ido desarrollando, como el 17-alilamino-17-demetoxigeldanamicina (17-AAG) (Figura 4), demostrando menos toxicidad y una notable actividad antitumoral. Sin embargo, su escasa solubilidad en agua provocó el desarrollo de nuevos análogos como el 17-dimetilaminoetilamino-17-demetoxigeldanamicina (17-DMAG) (Figura 4). Además de ser más

soluble en agua, muestra una actividad antitumoral mucho más potente [241, 186]. Numerosos inhibidores de HSP90 estimulan la activación de HSF1, induciendo por lo tanto una respuesta al choque térmico (Figura 3) [186]. El efecto pleiotrópico del bloqueo del sitio de unión al ATP de HSP90 por inhibidores como las ansamicinas es dual. Por un lado se consigue la inducción de la síntesis de HSPs (especialmente HSP70), mediante la competición por la unión con HSP90, lo cual induce la disociación de HSF1 del complejo con HSP90 y otras cochaperonas, que le mantienen en el citosol de manera inactiva. Por otro lado, se consigue la degradación de numerosas proteínas cliente de HSP90 a través de una vía dependiente de ubiquitín-proteosoma [278]. Dentro de las diferentes proteínas clientes modificadas por los inhibidores de HSP90, están STAT e IKK, así como MEK o diferentes subunidades de la NADPH oxidasa (Figura 5).

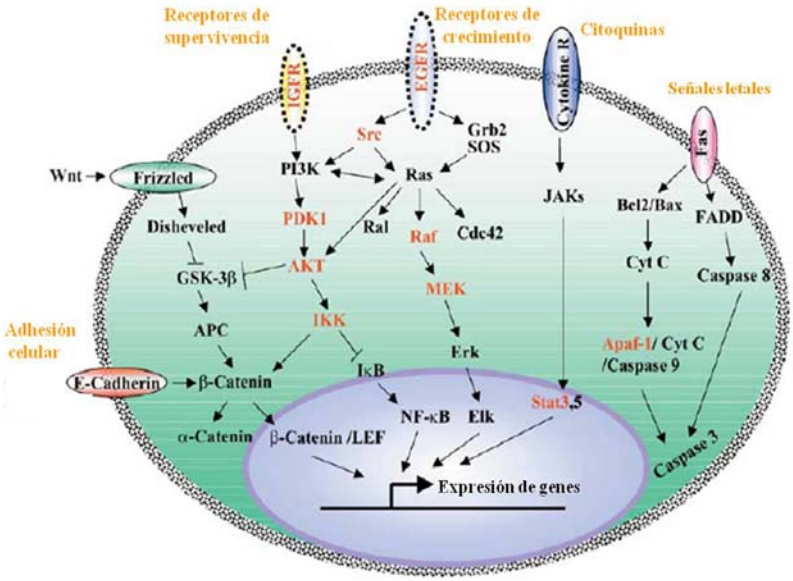


Figura 5. Diferentes rutas de señalización moduladas por los inhibidores de la HSP90 (modificado de Zhang et al [278]). Las proteínas cliente de HSP90 están marcadas en naranja.

1.2.1.2. HSP70

Las proteínas más estudiadas dentro de la familia de las HSPs citoplasmáticas de 70 kDa son HSC70, expresada de manera constitutiva, y HSP70, de forma inducible. Sus funciones chaperonas principales son; la ayuda en el ensamblaje de proteínas nativas y el re-ensamblaje de proteínas desnaturalizadas. Es interesante resaltar que en los casos en los que estas proteínas no pueden ser eficientemente re-ensambladas, HSP70 favorece su degradación. Un proceso clave responsable de la generación y el mantenimiento del correcto ensamblaje proteico es la red de proteostasis (PN) (Figura 6). La PN posee dos vías fundamentales que conducen al ensamblaje o la degradación mediada por ubiquitina, dando lugar al Yin y al Yang de la proteostasis (Figura 6). Aunque, la comprensión actual de este proceso es limitada [27], varios estudios sugieren un papel clave tanto de HSP70 como HSP90 en el mantenimiento de este equilibrio en escenarios patológicos, en los cuales los niveles de proteínas desensambladas aumentan considerablemente.

El equilibrio entre el ensamblaje (Yang) y la vía degradativa (Yin) está íntimamente ligado a los ciclos ATPasa de HSP70/HSP90, los cuales son regulados a través de la unión del ATP y las cochaperonas activadoras de la ATPasa (ATP a ADP). El principio operativo de la PN [185] sugiere que aunque determinadas aproximaciones pueden resolver los defectos en ensamblaje y tráfico en un compartimento celular, es necesario solventar estos problemas de manera general a través de la modulación del PN. El papel de HSP70 en la degradación proteica parece estar ligado a sus cochaperonas, como la proteína que interacciona con el extremo C-terminal de CHIP y la proteína atanógena asociada a BCL-2 (BAG-1). Así BAG-1 y CHIP, junto con HSP70, pueden cooperar para cambiar la actividad del sistema de la chaperona de degradación hacia el ensamblaje.

Figura 6. El Yin y el Yang de la proteostasis (modificado de Hutt* et al *[93]). *Representación esquemática del equilibrio entre las dos ramas de la red de la PN, el ensamblaje (Yang) y degradación (Yin), que manejan globalmente el ensamblaje proteico celular en condiciones fisio y patológicas. HSP70 y HSP90, componentes fundamentales de la PN, se encuentran como forma activa e inactiva en función de sus cochaperonas regulatorias. Forma competente HSP90 ($HSP90^{A}$, unida a ATP), inactivación de HSP90 ($HSP90^{I}$), forma activa de HSP70 ($HSP70^{A}$, unida al ADP).*

1.2.1.3. HSP27

HSP27 es una proteína ubicua cuya expresión es inducida por diversos tipos de estrés, tanto fisiológicos como ambientales. Se le considera una potente chaperona cuya función principal es la prevención de la acumulación de agregados proteicos. La organización de HSP27 parece ser crucial para el control de su actividad, pudiendo formar grandes oligómeros de hasta 800 kDa. El dímero parece ser la unidad básica de estos multi-complejos. La oligomerización es un proceso altamente dinámico que depende del estado de fosforilación de la proteína, induciendo modificaciones tanto en el tamaño del oligómero como en la actividad chaperona [174]. Además, HSP27 estimula la actividad catalítica de la maquinaria del proteosoma del 26S e induce la degradación de las proteínas ubiquitinadas en respuesta a estrés. A diferencia de HSP70, HSP27 interacciona directamente con la subunidad del proteosoma del 19S y se asocia a la ubiquitina, participando en la limpieza de proteínas que necesitan ser degradadas, contribuyendo de esta forma a su papel citoprotector [62].

1.2.2. El sistema de la Tiorredoxina

Los miembros de la familia de la TRX además de estar implicados en el ensamblaje proteico, tienen un papel fundamental en el mantenimiento del estado redox celular. El sistema de la TRX está basado en la reducción de grupos tiol y está compuesto fundamentalmente por la TRX, la reductasa de TRX, la proteína que interacciona con TRX [(también conocida como proteína de unión a la TRX (TRXBP) o proteína-1 sobre-expresada con vitamina D3 (VDUP-1)] y la Peroxiredoxina de la TRX (PRDX) [187] (Figura 7). El sistema de la TRX funciona activamente en la vasculatura y está presente en monocitos/macrófagos [209, 79], CMLVs [193, 183] , células endoteliales [265, 100], neutrófilos [145, 146] y eritrocitos [31, 146]. La homeostasis redox celular está regulada por la acción coordinada de numerosos mecanismos incluyendo el GSH, la familia de la TRX y la NADPH [266] (Figura 7). Los dos primeros son sistemas reductores de grupos tiol que tienen un papel clave en la defensa frente a la excesiva generación de ROS, así como en la regulación de la señalización de procesos tales como la inflamación, la proliferación celular y la apoptosis [85, 188, 263]. Estas moléculas mantienen el medio intracelular en un estado reducido. El GSH es usado por la GSH peroxidasa para reducir peróxidos, produciendo GSH disulfito o GSSG. La GSH reductasa reduce el GSSG a GSH reducido. Las propiedades antioxidantes de TRX tienen lugar mediante la acción de la PRDX. Esta puede interaccionar y modular la actividad de la NADPH oxidasa mediante la inactivación del H_2O_2 y su vía de señalización [120] (Figura 7). Bajo diversas condiciones de estrés, TRX y PRDX podrían ser liberados al medio extracelular [200, 208].

Debido a que la generación de ROS juega un papel clave en el desarrollo de la lesión aterosclerótica, antioxidantes como la TRX o la PRDX podrían actuar como agentes protectores en la respuesta frente al estrés oxidativo en la aterosclerosis.

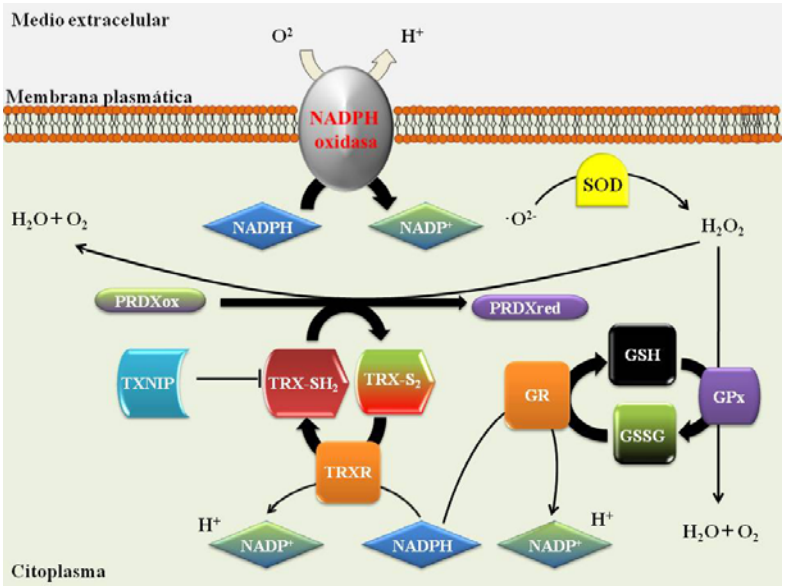


Figura 7. Mecanismo de acción de la familia de la TRX. *Efectos antioxidantes de la TRX dependientes de la PRDX en condiciones oxidativas. La TRX reducida recicla el H_2O_2 a través de PRDX. La TRX oxidada es entonces reducida por la TRX reductasa en presencia de NADPH. PRXred (PRDX reducida), PRDXox (PRDX oxidada), TRX-SH2 (TRX reducida), TRX-S2 (TRX oxidada), TRXR (TRX reductasa), GR (glutation reductasa), GPx (glutation peroxidasa).*

1.2.2.1. Funciones de la Tiorredoxina intracelular

Las TRXs son proteínas redox de pequeño peso molecular (aprox. 12 kDa) presentes en todo tipo de células procarióticas y eucarióticas siendo esenciales para la viabilidad celular. El ratón que carece de TRX es letal, lo cual indica la importancia funcional de TRX [225]. Hasta el momento, 3 variantes de la TRX humana han sido caracterizadas, codificados por genes diferentes. TRX1 es la más estudiada, se localiza en el citosol aunque puede translocar bajo condiciones de estrés a otros compartimentos celulares como el núcleo [265] o a la membrana [265]; TRX2 es la forma mitocondrial; y la SpTRX se expresa abundantemente en espermatozoides.

La TRX es una molécula antioxidante con propiedades únicas, ya que interacciona directamente con varias moléculas de señalización y factores de transcripción, modulando de esta forma diversos procesos tales como la proliferación o la apoptosis. Las proteínas que interaccionan con TRX incluyen a la señal de apoptosis regulada por la cinasa 1, TXNIP o factores de transcripción como NF-ϰB y la proteína activadora-1. TRX se expresa abundantemente en células endoteliales y aumenta sus niveles en situaciones de estrés oxidativo, posiblemente como respuesta al incremento en los niveles de ROS. TRX protege a las células de la vasculatura frente al H_2O_2 [161], regula la expresión de la hemo-oxigenasa-1 (HO-1, también conocida como HSP32) [244] e induce la MnSOD en la mitocondria [43]. Además desempeña un papel protector frente al estrés producido por NO en células endoteliales, pudiendo regular la actividad de NOS [279] y participar en la regulación del NO a través de múltiples mecanismos [220]. El estado redox celular puede influenciar la localización subcelular de TRX, ya que TRX puede encontrarse en forma oxidada en el núcleo de células en crecimiento exponencial, mientras que en las quiescentes se encuentran en el citoplasma [230]. Se ha descrito recientemente que la sobreexpresión de TRX en ratón protege a la placenta frente al estrés oxidativo y el crecimiento fetal mediante el aumento de la disponibilidad de glucosa [245]. Además, la administración de TRX es beneficiosa en la reducción del daño cerebral por isquemia/reperfusión al disminuir el área infartada a través de las propiedades redox de TRX [75].

1.2.2.2. Funciones de la Peroxirredoxina intracelular

Dentro de la superfamilia de TRX, las PRDXs son muy abundantes (entorno al 0,1-1 % del total de proteína soluble en células de mamíferos) y se encuentran de manera ubicua en todos los organismos [200, 264]. Las PRDXs son enzimas específicas de grupos tiol que no contienen selenio y que usan sus sitos activos redox de cisteínas para reducir los peróxidos y eliminar las ROS, reciclando el H_2O_2 mediante la reducción de varios hidroperóxidos en su correspondiente agua y alcohol. Las células de mamíferos expresan seis isoformas de PRDX (PRDX 1-6), que pueden estar localizados en el citosol (1, 2, y 6), la mitocondria (3 y 5), y en el retículo

endoplasmático (4) [32, 203]. Todas las isoformas contienen un residuo cisteína conservado que es el sitio primario de oxidación por H_2O_2. Dos mecanismos pueden ser responsables de la inactivación temporal de PRDX; su fosforilación por la cinasa dependiente de ciclina B/AKT y la hiperoxidación de su sitio activo [270]. El estado redox de la PRDX puede modificar asimismo su función ya que la hiperoxidación estimula su actividad chaperona [19]. Por ejemplo, frente a bajas concentraciones de H_2O_2, que se producen en condiciones de homeostasis celular, la PRDX tiende a formar oligómeros de bajo peso molecular, que además de presentar actividad peroxidasa protege a las proteínas de la degradación. Sin embargo, ante cambios notables en la concentración de H_2O_2 la PRDX experimenta cambios estructurales y tiende a formar oligómeros de alto peso molecular y adquiere la actividad chaperona [132, 201]. Su expresión puede verse modificada por estímulos prooxidativos como el LPS o ésteres de forbol [79, 261].

PRDX es un regulador importante de la homeostasis celular frente al H_2O_2 [32]. En células estimuladas con el factor de crecimiento derivado de plaquetas o con TNF-α, la sobreexpresión o el silenciamiento de PRDX, provoca respectivamente la reducción o el incremento de los niveles de H_2O_2 [40]. PRDX elimina el H_2O_2 de una manera mucho más eficiente que otros sistemas, como por ejemplo la catalasa, debido a que posee mucha mayor afinidad por el H_2O_2 que esta. Además de reducir H_2O_2, PRDX reduce los niveles de peroxinitritos a través de las reductasas de peroxinitritos [247].

1.2.2.3. Funciones de la TRX/PRDX extracelular

Además de su papel como chaperonas y como moléculas antioxidantes, TRX/PRDX pueden tener diferentes funciones dependiendo de su localización celular, lo que se ha denominado como la teoría de las "Moonlighting proteins" [97]. Esta teoría sustenta la idea de un gen=una proteína=una función se ha quedado muy simple teniendo en cuenta el gran número de proteínas que poseen dos o más funciones y todo ello dependiendo de su localización celular. Esta hipótesis no tiene por qué cubrir a todas las proteínas pero parece ser adecuada en el caso de TRX/PRDX. Sin embargo sus mecanismos de transporte no han sido completamente elucidados.

La TRX extracelular está presente en la circulación y sus niveles se incrementan debido al estrés oxidativo o a la inflamación [112]. La expresión de TRX se incrementa rápidamente y se secreta por células normales y tumorales aunque en este caso su secreción no parece seguir la clásica ruta del aparato de Golgi [208]. La función de TRX se regula mediante la unión de TXNIP [104]. El sitio redox activo de TRX media esta interacción, lo que lleva a la reducción en la actividad de TRX, sugiriendo que la asociación entre TRX-TXNIP puede ser un mecanismo importante en la regulación de estado redox celular, ya que además TXNIP sirve como transportador de TRX

del citoplasma hacia la membrana bajo condiciones de estrés oxidativo [265]. Por ejemplo, la hiperglicemia en CMLVs estimula la síntesis de TXNIP dando lugar a la inhibición de la actividad de TRX. Además, los animales diabéticos poseen mayor expresión vascular de TXNIP [215]. Por otro lado, bajo condiciones de estrés biomecánico en células cardiacas la expresión de TXNIP se ve reducida, aumentando la actividad de TRX [252]. Estos datos sugieren que TXNIP puede jugar un papel clave en las enfermedades cardiovasculares, funcionando como un sensor para el estrés biomecánico y oxidativo. Existe además una forma truncada de TRX que comprende los 80 u 84 aminoácidos del extremo N-terminal (TRX80) y es secretada, estando presente en el plasma donde fue originalmente purificada e identificada como factor estimulante de la citotoxicidad de eosinófilos [45]. La TRX80 recombinante se ha descrito como una citoquina mitogénica muy potente en células mononucleares de sangre periférica (PBMCs) un efecto que no posee TRX [178]. TRX80 difiere enormemente de TRX ya que forma un dímero que no posee actividad reductasa. La principal diana de TRX80 son los PBMCs a los cuales los dirige hacia una respuesta tipo linfocito cooperador-1 (Th1) vía la producción de IL-12 [177].

Existen menos datos disponibles sobre los niveles **extracelulares de PRDX**. PRDX-1 se encuentra en células endoteliales (ECs) dentro del aparato de Golgi [159], y la estimulación con PMA induce su translocación a la membrana plasmática [125]. La PRDX podría ser secretada por células de cáncer no microcítico de pulmón, posiblemente a través de una vía no clásica [33, 34]. Sin embargo, la función extracelular de PRDX-1 es desconocida. Muchas proteínas relacionadas con estrés oxidativo, incluyendo TRX/PRDX y HSPs, son liberadas al medio extracelular por células estresadas, transformadas y muertas, actuando como un sistema endógeno de alerta mediante la unión de estas señales a determinados receptores/sensores [10, 90, 137, 272]. Muchas de estas señales endógenas son reconocidas por el receptor TLR4 [10, 137]. Igualmente, PRDX-1 extracelular se una a TLR-4 y estimula la liberación de citoquinas pro-inflamatorias en macrófagos y células dendríticas, lo que sugiere que podría actuar como una molécula asociada a patrones de reconocimiento de daño. Su transporte podría depender de la unión a PKC mediante microvesículas [256].

Otro mecanismo que puede estar envuelto en la secreción activa de TRX y PRDX son los **exosomas,** ya que estudios proteómicos han descrito la presencia de TRX y PRDX en exosomas derivados de células B [28], células cancerosas de vejiga [255], células de cáncer colorrectal [39], de TRX además en orina [65] y de PRDX-1 en células de cáncer de pecho [234], leche materna humana [2] y saliva [66]. Las micropartículas son una población heterogénea de pequeñas vesículas cubiertas de membrana liberadas por numerosos tipos celulares bajo activación o apoptosis. La generación de micropartículas parece ser un proceso bien regulado, aunque estas vesículas difieren bastante en

tamaño, función y composición. A pesar de haber sido consideradas inicialmente como debris celular sin función celular específica, datos recientes demuestran un importante papel patofisiológico de los mecanismos orquestados en enfermedades vasculares. Dentro de las micropartículas, se encuentran unas vesículas más pequeñas denominadas exosomas que pueden participar también en el desarrollo de las enfermedades vasculares. El papel mecanístico de los exosomas en la mediación de enfermedades vasculares indica que pueden representar nuevas rutas en la señalización celular paracrina de la vasculatura.

Actualmente, la investigación del papel de las CSPs en aterosclerosis en un tema muy importante, aunque los mecanismos moleculares que subyacen en la función de las CSPs en la formación y/o rotura de la placa de ateroma deben ser dilucidados todavía.

II. OBJETIVOS

El objetivo general de esta tesis fue el estudio de la implicación de las CSPs en la aterosclerosis, ya que se ha observado que estas están presentes en la lesión aterosclerótica y pueden modular diversos procesos implicados en la misma. Además, se ha descrito que las CSPs pueden ser secretadas al medio extracelular en enfermedades de tipo inflamatorio-oxidativo como la aterotrombosis. El conocimiento de las funciones que estas CSPs tienen en el origen y desarrollo de la aterosclerosis pueden aportar importante información para el impulso de nuevas terapias.

Los objetivos específicos planteados fueron los siguientes:

1. Examinar la expresión de HSP90 y HSP70 en la placa humana de aterosclerosis avanzada.

2. Estudiar los efectos de la inhibición de HSP90, mediante dos inhibidores específicos de HSP90 (17-AAG, 17-DMAG), en la respuesta inflamatoria de la aterosclerosis experimental.

3. Analizar los efectos de la inhibición de HSP90 mediante el 17-DMAG en el estrés oxidativo y en la diferenciación de monocito-macrófago en aterosclerosis experimental.

4. Determinar los niveles plasmáticos de PRDX-1/TRX en pacientes con estenosis carotídea y en sujetos asintomáticos con un grosor de la íntima-media conocido.

5. Estudiar los mecanismos de tráfico intracelular de PRDX-1/TRX y la potencial asociación de este transporte con el incremento en la actividad NADPH oxidasa.

III. MATERIALES, MÉTODOS Y RESULTADOS

3.1. Los inhibidores de HSP90 atenúan la respuesta inflamatoria en aterosclerosis.

En el presente trabajo se han desarrollado los objetivos 1 y 2 de la tesis para lo cual estudiamos en primer lugar la expresión y distribución de HSP90 y HSP70 en 60 placas humanas de aterosclerosis avanzadas. La atenuación de la capa fibrosa en un determinante clave para la estabilidad de la placa aterosclerótica [198], ya que confiere resistencia a la rotura debido a su composición en colágeno y otras proteínas de la matriz extracelular sintetizadas por CMLVs. Las células inmuno-inflamatorias son capaces de liberar proteasas que degradan la matriz extracelular y promueven la inestabilidad y la rotura de la placa. Además, el número de células infiltrantes y la expresión de mediadores inflamatorios están incrementados en las zonas proclives a la rotura en las placas humanas de aterosclerosis avanzada [142]. Durante el proceso de elaboración de este trabajo otros autores publicaron un artículo en el que describían la sobreexpresión de HSP90 en las placas y suero de pacientes con aterosclerosis en comparación con sujetos control, lo que podría contribuir potencialmente a la inestabilidad de la placa mediante la inducción de la respuesta inmune [29]. Nosotros ampliamos estos datos y describimos un aumento de la expresión de HSP90 en la zona inflamatoria de la placa, caracterizada por una delgada capa fibrosa. Estas placas son las que tienen mayor tendencia a la rotura y, por tanto, a provocar eventos clínicos. Por el contrario, la tinción para HSP70 se asoció en mayor medida a la zona fibrosa de las placas con una capa fibrosa gruesa. Para continuar el desarrollo del segundo objetivo, una vez comprobada la presencia diferencial de HSP90 y HSP70 en la placa aterosclerótica, procedimos a analizar el uso de inhibidores de HSP90 en aterosclerosis experimental y su efecto modulador en la respuesta inflamatoria en aterosclerosis. HSP90 es una chaperona ubicua implicada en el ensamblaje, activación y maduración de numerosas proteínas, denominadas proteínas cliente. El uso de estos inhibidores de HSP90 tiene efectos pleiotrópicos como son la modulación de diversas rutas de señalización, debido a la degradación de las proteínas cliente de HSP90, y el aumento en la síntesis de diferentes HSPs, como HSP70. Mediante estudios *in vitro* con células implicadas en el desarrollo de la placa de ateroma (monocitos/macrófagos y CMLVs) demostramos que la inhibición de HSP90, mediante el uso de inhibidores específicos su actividad como son el 17-AAG y el 17-DMAG, incrementó los niveles de HSP70. Asimismo, redujeron la activación de los factores de transcripción STAT3 y NF-$\varkappa$B y los niveles intra y extracelulares de MCP-1 e IL6 inducidas por un cóctel de citoquinas pro-inflamatorias (IFN-γ e IL-6). Para comprobar la relevancia de nuestros resultados *in vitro*, se llevaron a cabo estudios *in vivo* en ratones hiperlipidémicos ApoE$^{-/-}$. Para este fin, se usó el 17-DMAG debido a su mayor efecto

anti-inflamatorio observado *in vitro*. El 17-DMAG redujo el tamaño total de la lesión a lo largo del arco aórtico y su contenido lipídico. Además, el tratamiento con este inhibidor de HSP90 redujo de manera significativa el contenido en macrófagos de la lesión, la activación de STAT3 y NF-κB, así como los niveles de MCP-1 locales y circulantes.

Así, la conclusión de este trabajo es que HSP90 se asocia a la inestabilidad de la placa humana de aterosclerosis avanzada, mientras que HSP70 se asocia a la estabilidad de la misma. Además, el uso de los inhibidores de HSP90 reduce la respuesta inflamatoria, el tamaño y el contenido lipídico de la lesión, lo que sugiere que HSP90 podría ser una nueva diana terapéutica en el tratamiento de enfermedades inflamatorias como la aterosclerosis.

Artículo publicado en *Cardiovascular Research*. 2010 May 1;86(2):330-7.

Cardiovascular Research Advance Access published March 10, 2010

Cardiovascular Research
doi:10.1093/cvr/cvq046

Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis

Julio Madrigal-Matute[1], Oscar López-Franco[1], Luis Miguel Blanco-Colio[1], Begoña Muñoz-García[1], Priscila Ramos-Mozo[1], Luis Ortega[2], Jesus Egido[1], and Jose Luis Martín-Ventura[1]*

[1]Vascular Research Lab, IIS, Fundación Jiménez Díaz, Autónoma University, Av. Reyes Católicos 2, 28040 Madrid, Spain; and [2]Department of Pathology, Hospital Clínico San Carlos, Madrid, Spain

Received 22 September 2009; revised 5 February 2010; accepted 8 February 2010

Time for primary review: 34 days

Aims Heat shock protein 90 (HSP90) is a ubiquitous chaperone involved in the folding, activation, and assembly of many proteins. HSP90 inhibitors [17-allylamino-17-demethoxygeldamycin (17-AAG)/17-dimethyl aminothylamino-17-demethoxygeldanamycin hydrochloride (17-DMAG)] bind to and inactivate HSP90, increasing the heat shock response and suppressing different signalling pathways. We aim to investigate the effect of HSP90 inhibitors in the modulation of inflammatory responses during atherogenesis.

Methods and results In human atherosclerotic plaques, HSP90 immunostaining was increased in inflammatory regions and in plaques characterized by lower cap thickness. In cultured human macrophages and vascular smooth muscle cells, treatment with either 17-AAG or 17-DMAG increased HSP70 expression and reduced transcription factor [signal transducers and activators of transcription (STAT) and nuclear factor-κB (NF-κB)] activation and chemokine expression induced by proinflammatory cytokines. *In vivo*, hyperlipidaemic ApoE$^{-/-}$ mice were randomized to 17-DMAG (2 mg/kg every 2 days, $n = 11$) or vehicle injected ($n = 9$) during 10 weeks. Atherosclerotic plaques of mice treated with 17-DMAG displayed increased HSP70 expression and diminished NF-κB and STAT activation, along with decreased lesion, lipid, and macrophage content, compared with vehicle-injected mice. In addition, treatment with 17-DMAG significantly reduced monocyte chemoattractant protein-1 levels, both in plaques and in plasma.

Conclusion HSP90 expression is associated with features of plaque instability in advanced human lesions. HSP90 inhibitors reduce inflammatory responses in atherosclerosis, suggesting that HSP90 could be a novel therapeutic target in atherosclerosis.

Keywords Atherosclerosis • Inflammation • Heat shock proteins

1. Introduction

Atherothrombosis is the leading cause of mortality in the Western world.[1] The underlying pathological process is a thickening of the arterial wall. However, it has been increasingly realized that lesions responsible for acute events may not necessarily be critically obstructive.[2] Attenuation of the fibrous cap is a main determinant of plaque stability[3] since the cap confers resistance to rupture due to its composition of collagen and other extracellular matrix (ECM) proteins, synthesized by vascular cells. Inflammatory cells are able to release different proteases, which lead to degradation of ECM proteins and promote plaque instability and rupture. Interestingly, the number of infiltrating cells and the expression of inflammatory mediators are increased in rupture-prone regions of culprit human atherosclerotic plaques.[4]

Heat shock protein 90 (HSP90) is a ubiquitous molecular chaperone that is involved in the folding, activation, and assembly of many proteins, including key mediators of signal transduction and transcriptional regulation.[5] It functions as part of a multichaperone complex via association with cochaperones (e.g. HSP70) and several client proteins [e.g. IκB kinase (IKK) and signal transducers and activators of transcription (STAT)]. Blocking the ATP-binding site of HSP90 by inhibitors such as ansamycins ultimately results in both up-regulation of HSP expression (specially HSP70) and degradation of some client

* Corresponding author. Tel: +34 915504800; fax: +34 915504399, Email: jlmartin@fjd.es

proteins via an ubiquitin–proteasome-dependent pathway.[6] Among the different client proteins modified by HSP90 inhibitors, STAT and nuclear factor-kB (NF-κB) signalling pathways play a critical role in mediating inflammatory and immune responses. Janus kinase (JAK)/STAT is an important signalling pathway that functions downstream cytokine receptors.[7] NF-κB activation requires phosphorylation of IκBα by IKK and IκBα degradation by the proteasome, allowing NF-κB to enter the nucleus. Both signalling pathways are activated by proatherogenic cytokines and control the expression of different proinflammatory genes, such as monocyte chemoattractant protein-1 (MCP-1) or regulated upon activation, normal T cell expressed and secreted (RANTES). In this respect, it has been demonstrated that both clinical and experimental therapies that decrease inflammation in atherothrombosis may have beneficial effects by preventing the progression of this disease.[8–10] Among other experimental therapies, it has been recently observed that modulation of both NF-κB and STAT decreases inflammatory processes in the ApoE$^{-/-}$ model of atherosclerosis.[9,10]

Although the main therapeutic application of HSP90 inhibitors is related to the field of cancer, it has been demonstrated that these drugs are also able to block the activity of certain proinflammatory mediators in different cell types.[11,12] Moreover, the HSP90 inhibitor 17-allylamino-17-demethoxygeldamycin (17-AAG) is able to attenuate inflammation in several diseases.[13–15] At present, there are no data regarding the effect of HSP90 inhibitors in cardiovascular diseases. In the present study, we hypothesized that HSP90 inhibitors may attenuate inflammatory processes associated with atherosclerosis. First, we performed an observational study to address the expression of HSP90 in human atherosclerotic plaques and its potential association with features of plaque instability. Furthermore, we analysed the intracellular mechanisms modified by HSP90 inhibitors, both *in vitro* (vascular cells) and *in vivo* (ApoE$^{-/-}$ experimental model).

2. Methods

2.1 Patients

Sixty consecutive patients undergoing carotid endarterectomy in our institutions were included in the study (69 ± 8 years, 26% women, 82% hypertensive, and 34% diabetics). Atherosclerotic plaques (stary stages V and VI) were fixed with paraformaldehyde and embedded in paraffin. The local committees on human research at Fundacion Jimenez Diaz-Autonoma University approved the study, which was performed in accordance with the principles outlined in the Declaration of Helsinki, and all participants gave written informed consent.

2.2 Cell culture

Human vascular smooth muscle cells (VSMC) were purchased from ATCC (CRL-1999) and maintained in HAM's F12 (BioWhittaker) supplemented with 10% FBS (BioWhittaker), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Cells were used between passages 3 and 7. Human THP-1 monocytic cell line was purchased from ATCC (CRL-1593) and cultured with RPMI 1640 (BioWhittaker) supplemented with 10% decomplemented FBS, 2 mM L-glutamine and antibiotics. THP-1 cells were differentiated to macrophages by incubation with 10^{-7} M PMA for 48 h.[16] For experiments, cells were pre-incubated with 0% FBS during 24 h.

2.3 Reagents

HSP90 inhibitors [17-AAG or 17-dimethyl aminothylamino-17-demethoxygeldanamycin hydrochloride (17-DMAG), Biomol] were diluted in saline at 10 mmol/L and stored at −20°C. A cytokine cocktail was composed of 100 U/mL of human interleukin 6 (IL6, PeproTech) and 1000 U/mL of human interferon gamma (IFNγ, PeproTech).

2.4 RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen). One microgram of RNA was used to perform the reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems). Real-time polymerase chain reactions (PCRs) were performed on ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to the manufacturer's protocol using the $\Delta\Delta C_t$ method as described.[17] Predeveloped primers and probe assays were obtained for human 18S, HSP70, HSP90, MCP-1, and IL6 from Applied Biosystems. Expression levels are given as a ratio to housekeeping gene 18S and data are expressed as fold vs. control values.

2.5 Western blot

Equal amount of total protein was separated on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Subsequently, membranes were blocked and incubated with mouse monoclonal anti-HSP70 (SPA-810, Stressgen), goat polyclonal anti-pSTAT3 (#9131, Cell Signaling), STAT3 (sc-8019, Santa Cruz Biotechnology), NF-κB p50 (sc-7178, Santa Cruz Biotechnology), rabbit polyclonal anti-IκBα (sc-371, Santa Cruz Biotechnology), or mouse monoclonal anti-α-tubulin (T-5168, Sigma-Aldrich). Proteins were visualized by ECL Western Blotting Detection Reagents (Amersham Biosciences) according to the manufacturer's instructions.

2.6 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) for NF-κB-binding activity was performed with nuclear protein extracts from cells as described.[17] The specificity of the assay was tested with a 100-fold excess of unlabelled NF-κB consensus oligonucleotide added to the ^{32}P-labelled probe-binding reaction.

2.7 Experimental atherosclerosis

Male ApoE$^{-/-}$ mice (12 weeks of age; Jackson Laboratory) were fed on a Western diet during 11 weeks. After 1 week at feeding, mice were randomized into two groups: DMAG treatment ($n = 11$) and control ($n = 9$). The treated group was ip injected with 2 mg/kg of 17-DMAG diluted in saline every 2 days during 10 weeks. Controls consisted on ApoE$^{-/-}$ mice injected with vehicle (saline). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by Fundación Jimenez Diaz Ethics Review Board.

2.8 Lipid determinations and enzyme-linked immunosorbent assay

Cholesterol and triglycerides were tested in serum samples from mice at fasting by a commercially available kit (TR13421 and TR0100, Sigma). Soluble MCP-1 levels were measured in the supernatants of cells after different experimental conditions and in mice serum with a commercially available enzyme-linked immunosorbent assay (ELISA; DCP00, R&D systems) following the manufacturer's instructions.

2.9 Immunohistochemistry

Paraffin-embedded human carotid atherosclerotic plaques were cross-sectioned into 4 μm-thick pieces, dewaxed, and rehydrated. Mouse monoclonal anti-HSP90 (SC-13119, Santa Cruz Biotechnology) and anti-HSP70 (SPA-810, Stressgen) antibodies were applied. Negative controls using the corresponding IgG were included for checking non-specific staining. Cap thickness was evaluated at three different points in the histology sections

by a pathologist blind to the clinical details. Thin caps were considered when the mean of these three measurements was <165 μm, as described previously.[18] Data of computer-assisted morphometric analysis were expressed as percentage of positive staining/mm^2 as described.[17]

Anaesthetized mice were saline perfused. Liver was paraffin embedded for histology. Aortic samples were frozen in OCT and serial 6 μm sections were stained with Oil red O/haematoxylin. In the root of each animal, maximal lesion size and lipid content were quantified by computerized morphometry and were averaged. In addition, to analyse the amount of lesion along the entire arch, lesion and lipid content were individually quantified as the sum of all measurements covering a total of 3000 μm. Data are presented as the mean of individual animal measurements in each group.

Activated NF-κB was detected by Southwestern histochemistry with digoxigenin-labelled probes, using competition and mutant probe as specificity controls.[9] Rat monoclonal anti-monocytes/macrophages (clone MOMA-2, MCA519, Serotec), goat polyclonal anti-MCP-1 (SC-1785, Santa Cruz Biotechnology), goat polyclonal anti-pSTAT3 (SC-7993, Santa Cruz Biotechnology), rabbit polyclonal anti-HSP90 (ab19021, Abcam), rabbit polyclonal anti-HSP70 (ab31010, Abcam), and rabbit polyclonal anti-RANTES (AB2109P, Millipore) antibodies were applied. In all cases, secondary antibodies and ABComplex/HRP were added and sections were stained with 3,3′-diaminobenzidine and mounted in Pertex. Quantification was performed by a pathologist blind to the experimental groups analysed. Immunohistochemistry data are expressed as percentage of positive staining/μm^2 as described.[9,10]

For colocalization studies in human plaques with VSMC and macrophages, double immunohistochemistry/immunofluorescence for HSP90 was carried out in serial sections along with α-actin (monoclonal CLONE 1A4, DAKO) and CD68 (monoclonal CLONE KP1, DAKO). For colocalization studies of HSP70, pSTAT3, and MCP-1 in mice plaques with macrophages (MOMA-2), double immunohistochemistry/immunofluorescence was performed and for VSMC (AB15734, Abcam), immunohistochemistry in serial sections was performed. For colocalization of NF-κB and macrophages/VSMC, immunohistochemistry for cells was performed after Southwestern *in situ* for NF-κB activation.

2.10 Statistical analysis

Statistics were performed using GraphPAD InStat (GraphPAD Software). *In vitro* experiments were performed at least three times. Results are expressed as mean ± SEM and were analysed by the Mann–Whitney non-parametric, Wilcoxon paired or Student's *t*-test when appropriate (two-tailed, significant differences at $P < 0.05$).

3. Results

3.1 HSP90 and HSP70 expression in culprit human carotid atherosclerotic plaques

We studied the expression and distribution of HSP90 and HSP70 in advanced human atherosclerotic plaques: the inflammatory region of the shoulder, characterized by a high macrophage accumulation, and the fibrous region, with increased VSMC and collagen content. Quantification of HSP90 and HSP70 immunostaining in 60 human atherosclerotic plaques showed an increased expression of HSP90 and HSP70 in the shoulder region in relation to the fibrous area (9.2 ± 1.1 vs. 6.6 ± 0.7% positive staining/mm^2, $P < 0.05$, and 5 ± 0.6 vs. 3.8 ± 0.5% positive staining/mm^2, P = n.s., respectively, *Figure 1A* and *B*). Interestingly, when atherosclerotic plaques were classified according to the cap thickness, we observed that plaques with thin caps (<165 μm, as described by Virmani and coworkers[18]) displayed higher total HSP90 but lower HSP70 levels than those

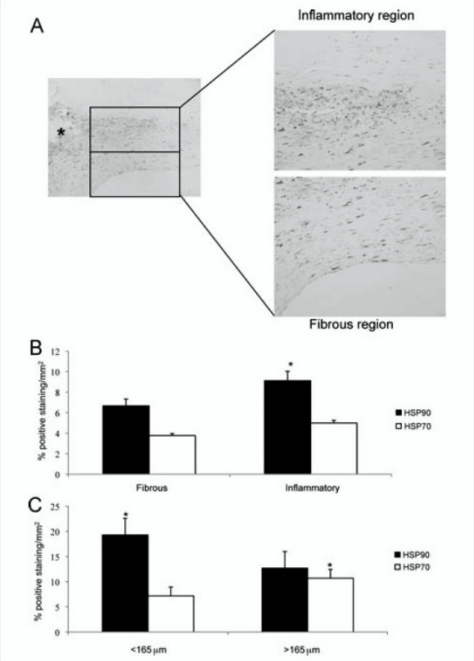


Figure 1 HSP90 and HSP70 immunostaining in human carotid atherosclerotic plaques. (*A*) Expression of HSP90 in the inflammatory and fibrous regions of the plaques. Atheroma is localized by an asterisk. Magnification ×100 (detail ×200). (*B*) Quantification of HSP90- and HSP70-immunostained area in different regions of carotid atherosclerotic plaques ($n = 60$, $^*P < 0.05$). (*C*) Quantification of total HSP90 and HSP70 immunostaining in plaques with cap thickness <165 or >165 μm.

plaques with caps >165 μm (19.3 ± 2.5 vs. 12.7 ± 1.5% positive staining/mm^2, and 7.2 ± 1.5 vs. 10.7 ± 2% positive staining/mm^2, respectively, $P < 0.05$ for both, *Figure 1C*).

Moreover, immunostaining for HSP90 and α-actin (VSMC) and CD68 (macrophages) in serial tissue sections showed that both macrophages and VSMC present in human plaques are able to express HSP90 (see Supplementary material online, *Figure S1*). These results suggest that whereas HSP90 could be a marker of instability, HSP70 is associated with features of stability, in advanced human atherosclerotic plaques.

3.2 HSP90 inhibitors modulate HSP70 levels and inflammatory signalling pathways in vascular cells

Treatment of human cells with either 17-AAG or 17-DMAG dose-dependently increased mRNA and protein expression of HSP70 in VSMC (data not shown) and macrophages (*Figure 2A* and *B*). In contrast, HSP90 expression was not significantly modified by drug treatment at the doses tested (*Figure 2A* and *B*). Furthermore, the effect of

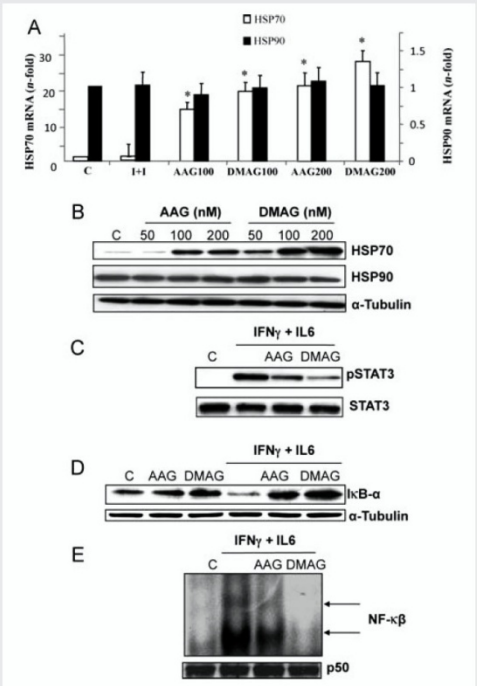


Figure 2 Effect of HSP90 inhibitors on HSP expression and client protein activation in vascular cells. (*A*) Real-time PCR and (*B*) western blot analysis of HSP70 and HSP90 in macrophages treated with different doses of 17-AAG or 17-DMAG for 4 h. (*C*) Western blot analysis of pSTAT3/STAT3 and (*D*) IκBα in VSMC and (*E*) EMSA of NF-κB and western of p50 in macrophages pre-treated for 4 h with 200 nM of 17-AAG or 17-DMAG and stimulated with IFNγ/IL6 for 30 min (pSTAT3) or 60 min (IκBα, NF-κB and p50). *$P < 0.05$ vs. basal.

HSP90 inhibitors on client proteins was analysed. Stimulation of VSMC with a cytokine cocktail (IL6 + IFNγ) induced STAT3 phosphorylation at 30 min and this effect was abolished by HSP90 inhibitor pre-treatment (*Figure 2C*). Similar results were obtained in macrophages (data not shown). In order to determine whether HSP90 inhibitors could interfere with NF-κB signalling pathway in our experimental conditions, IκBα and DNA-binding activity were studied by western blot and EMSA, respectively. As observed in *Figure 2D*, the diminution of IκBα levels induced by cytokines was prevented by 17-AAG/17-DMAG pre-treatment. Accordingly, pre-incubation of cells with 17-AAG or 17-DMAG decreased cytokine-induced NF-κB activation (*Figure 2E*).

3.3 HSP90 inhibitors decrease cytokine levels in vascular cells

Monocyte recruitment to the vascular lesion is mainly mediated by MCP-1. Since this chemokine is transcriptionally regulated by NF-κB, we studied the effect of HSP90 inhibitors on MCP-1 levels.

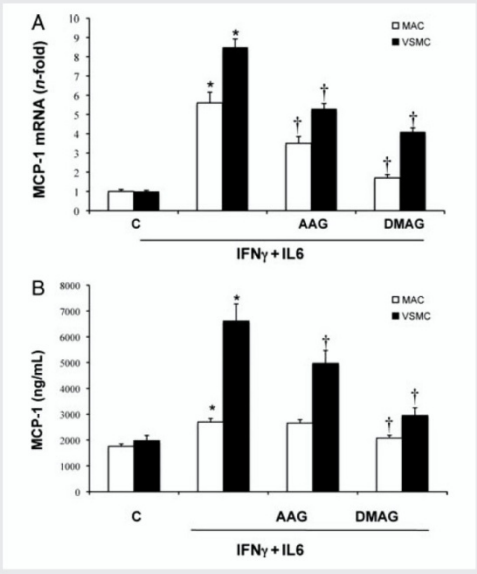


Figure 3 Effect of HSP90 inhibitors on MCP-1 levels in vascular cells. (*A*) Real-time PCR of MCP-1 mRNA in VSMC and macrophages pre-treated for 4 h with 17-AAG and 17-DMAG (100 and 200 nM) and stimulated with IFNγ/IL6 for 3 h. (*B*) ELISA of soluble MCP-1 in conditioned media of macrophages and VSMC pre-treated for 4 h with 17-AAG and 17-DMAG (200 nM) and stimulated with IFNγ/IL6 for 24 h (*$P < 0.05$ vs. basal, †$P < 0.05$ vs. IFNγ/IL6).

Incubation of VSMC and macrophages with cytokines for 3 h increased MCP-1 mRNA expression and 17-AAG/17-DMAG dose-dependently prevented this effect (*Figure 3A*). Similarly, HSP90 inhibitors decrease IL6 expression (% of reduction vs. stimulus: 17-AAG, 39 ± 9%; 17-DMAG, 69 ± 5% in VSMC, and 17-AAG, 36 ± 5%; 17-DMAG, 90 ± 15% in macrophages, $P < 0.05$ for all). In addition, we observed a decrease in MCP-1 levels in conditioned media of cells pre-treated with HSP90 inhibitors (*Figure 3B*).

3.4 17-DMAG decreases inflammatory cell infiltration in experimental atherosclerosis

To test the relevance of our *in vitro* results, *in vivo* studies were performed in hyperlipidaemic ApoE$^{-/-}$ mice. For that purpose, 17-DMAG was used due to its higher anti-inflammatory effects observed *in vitro*. The dose of the drug was chosen based on previous papers.[19,20] Mice received 0.2 mL ip administration of 2 mg/kg 17-DMAG three times a week on alternate days, since after 48 h, these drugs are cleared from blood.[21] Systemic effects of 17-DMAG treatment were evaluated in liver from ApoE$^{-/-}$ mice. Hepatic morphology was normal, without signs of inflammation, necrosis, or hepatocyte alterations (data not shown). Furthermore, 17-DMAG did not modify serum levels of cholesterol and triglycerides (1224 ± 392 vs. 1304 ± 496 mg/dL and 96 ± 15 vs. 81 ± 25 mg/dL, respectively, $P > 0.05$ vs. control group).

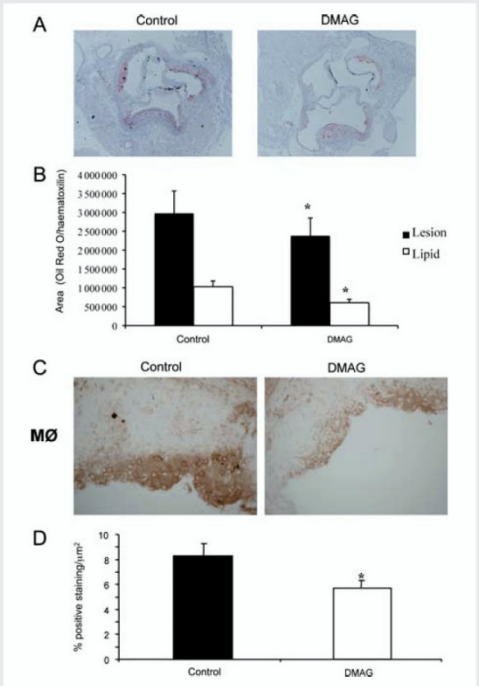


Figure 4 Effect of 17-DMAG on lesion size and macrophage content in ApoE $^{-/-}$ mice. (*A*) Representative images of Oil-red/haematoxylin staining in aortic lesions of control and 17-DMAG treated mice (magnification ×25). (*B*) Quantification of the lesion and lipid content in the entire arch of ApoE $^{-/-}$ mice ($n = 20$, $^{*}P < 0.05$ vs. control). (*C*) Representative images of macrophages (MØ) in control and 17-DMAG-treated mice (magnification ×100). (*D*) Quantification of MOMA levels in the ApoE $^{-/-}$ experimental model ($n = 20$, $^{*}P < 0.05$ vs. control).

17-DMAG reduced the maximal aortic lesion size in the root (357 ± 24 vs. $432 \pm 33 \times 10^3$ µm^2, $P = 0.08$) and the lipid content (116 ± 17 vs. $158 \pm 31 \times 10^3$ µm^2, $P = 0.07$) compared with vehicle-injected mice, although it did not reach statistical significance. However, when we analysed the total amount of lesion along the entire arch (3000 µm) and their lipid content, we observed that 17-DMAG treated animals showed a significant reduction in both parameters (2376 ± 667 vs. $2973 \pm 735 \times 10^3$ µm^2 and 605 ± 187 vs. $1028 \pm 315 \times 10^3$ µm^2, respectively, $P < 0.05$ for both, *Figure 4A* and *B*). Interestingly, treatment with 17-DMAG significantly reduced the macrophage content of lesions (5.4 ± 0.6 vs. $8.6 \pm 1\%$, $P < 0.05$, *Figure 4C* and *D*).

3.5 17-DMAG decreases inflammatory signalling pathways in lesions of ApoE$^{-/-}$ mice

To understand the potential mechanisms involved in the diminution of macrophage infiltration observed in 17-DMAG treated mice, we studied the effect of 17-DMAG on HSP70 expression and on the activation of its client proteins STAT3 and NF-κB. We observed that 17-DMAG increased HSP70 expression compared with vehicle-injected mice (6.8 ± 0.8 vs. $4.7 \pm 0.5\%$, $P < 0.05$, *Figure 5*). In contrast, HSP90 levels were not modified by 17-DMAG treatment (2.9 ± 0.6 vs. $3.3 \pm 0.9\%$, $P > 0.05$, data not shown). In addition, untreated mice displayed a higher staining for pSTAT3 than 17-DMAG treated mice (6.1 ± 1.1 vs. $2.8 \pm 0.7\%$, $P < 0.05$, *Figure 5*). Moreover, untreated mice showed stronger nuclear staining for NF-κB compared with 17-DMAG-treated mice (*Figure 5*). Nuclear staining was not observed with unlabelled consensus competition. The percentage of NF-κB-positive cells decreased in aortas from 17-DMAG-treated mice compared with vehicle-injected mice (1.9 ± 0.4 vs. $3.6 \pm 0.6\%$, $P < 0.05$, *Figure 5*).

Finally, HSP70 and pSTAT3 expression, as well as NF-κB activation, colocalized with VSMC and macrophages in mice plaques (see Supplementary material online, *Figure S2*).

3.6 17-DMAG decreases chemokine levels in ApoE$^{-/-}$ mice

To finally test the potential anti-inflammatory effect of 17-DMAG, chemokine expression was determined in atherosclerotic plaques of ApoE$^{-/-}$ mice. As observed in *Figure 6*, MCP-1 immunostaining was decreased in 17-DMAG-treated mice compared with vehicle-injected mice (3 ± 0.7 vs. 5.5 ± 0.3, $P < 0.05$). Both VSMC and macrophages expressed MCP-1 in mice plaques (see Supplementary material online, *Figure S2*). Similarly, 17-DMAG treatment decreased RANTES expression (4.4 ± 0.4 vs. $6.1 \pm 0.7\%$, $P < 0.05$, data not shown). In agreement, 17-DMAG diminished MCP-1 serum levels (35 ± 4 vs. 62 ± 5 ng/mL, $P < 0.05$, *Figure 6C*).

4. Discussion

It is well established that the breakdown of atherosclerotic plaques occurs more frequently in thin cap atherosclerotic plaques and where there is a great amount of inflammatory cells. Studies on coronary arteries of patients suffering myocardial infarction demonstrated that the rupture of atheroma usually takes place in the shoulder region,[22] an area characterized by a high inflammatory content, NF-κB activation, and MCP-1 expression.[4,23] In this study, we observed strong HSP90 immunostaining in advanced human atherosclerotic plaques, preferentially in the shoulder region. Moreover, HSP90 levels were higher in those plaques where the fibrous cap was thinner, suggesting that HSP90 plays an important role in the instability of advanced human atherosclerotic plaques. It has been very recently reported that HSP90 is overexpressed both in plaque and serum from patients with atherosclerosis, potentially contributing to plaque instability by inducing an immune response.[24] Our data extend these recent findings and suggest HSP90 as a possible therapeutic target in atherosclerosis.

Although the inhibitors of HSP90 are of therapeutic interest primarily in cancer,[25] evidence is emerging for the potential beneficial role of HSP90 inhibitors in the treatment of other inflammatory diseases, such as rheumatoid arthritis,[26] endotoxin-induced uveitis and murine sepsis.[14,15] Whereas initial inflammatory response could help to prevent lipid accumulation inside the atherosclerotic plaques, a chronic inflammatory response is associated with plaque progression. For that reason, strategies that prevent this pathological

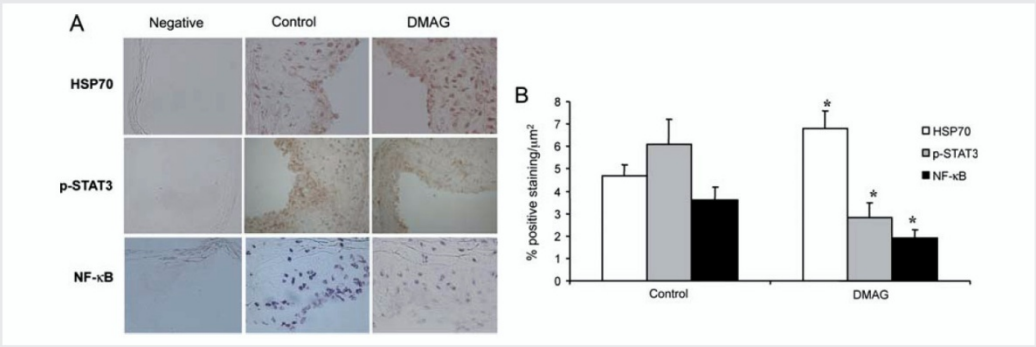


Figure 5 Effect of 17-DMAG on HSP70 and client proteins in vascular lesions of ApoE $^{/}$ mice. (*A*) Representative images of HSP70, pSTAT3 levels, and NF-κB activation in control and 17-DMAG-injected mice (magnification ×100). (*B*) Quantification of HSP70 (white), pSTAT3 (grey), and NF-κB activation (black) positive staining in aortic samples of ApoE $^{/}$ mice ($n = 20$, $^{*}P < 0.05$ vs. control). Negative controls were performed with IgG isotype (for HSP70 and pSTAT3) or unlabelled consensus oligonucleotide (for NF-κB).

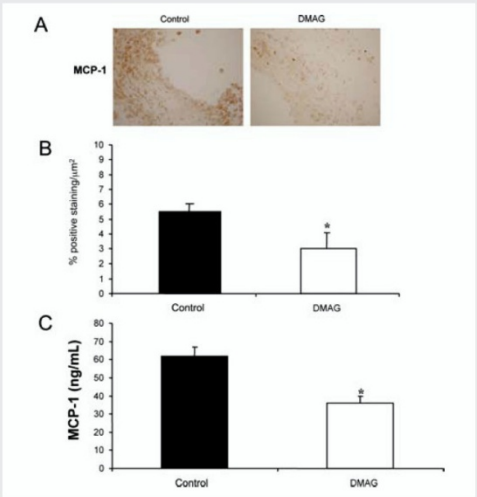


Figure 6 Effect of 17-DMAG on chemokine levels in ApoE $^{/}$ mice. (*A*) Representative images of MCP- 1 in control and 17-DMAG-injected mice (Magnification ×100). (*B*) Quantification of MCP-1 immunostaining in the ApoE $^{/}$ experimental model ($n = 20$, $^{*}P < 0.05$ vs. control). (*C*) Quantification of MCP-1 serum levels in ApoE $^{/}$ mice ($n = 20$, $^{*}P < 0.05$ vs. control).

response would be of potential benefit. In this respect, the beneficial effect of HSP90 inhibitors in inflammatory diseases could be due to their double activity: degradation of client proteins involved in different inflammatory signalling pathways and up-regulation of anti-inflammatory HSP expression (especially HSP70). We have observed that HSP90 inhibitors could diminish the expression and activation of inflammatory mediators both *in vitro* and *in vivo*. However, further studies are needed to clarify whether the reduced inflammatory response observed with HSP90 inhibitors is due to HSP70 up-regulation, inhibition of HSP90 activity, or both.

Among the different client proteins of HSP90 involved in inflammatory diseases, STAT and NF-κB are the most representative examples. JAK/STAT is an important signalling pathway that functions downstream cytokine receptors and regulates the initiation/progression of atherosclerosis and the remodelling in response to injury.[7,27] JAK/STAT activation has been previously found in cultured vascular cells under inflammatory conditions and in atherosclerotic lesions.[10,28,29] Fatty streak formation was reduced in STAT3 conditional knockout mice when compared with their wild-type littermates.[28] In contrast, STAT3 up-regulation by antisense oligodeoxynucleotides therapy targeting its negative regulator (suppressors of cytokine signalling 3) increases inflammatory responses in the ApoE$^{-/-}$ model of atherosclerosis.[10] These results are in agreement with the present paper since we have observed that prevention of STAT3 activation by HSP90 inhibitors is able to decrease inflammatory responses in vascular cells and atherosclerotic plaques.

NF-κB signalling plays a critical role in mediating inflammatory and immune responses. Activation of NF-κB requires phosphorylation of IκB by IKK and degradation by the proteasome, allowing NF-κB to enter the nucleus to transcriptionally regulate the expression of different proinflammatory genes (e.g. MCP-1). Since IKK exists in complexes with HSP90, disruption of these complexes by HSP90 inhibitors blocks IKK function and, consequently, NF-κB activation. We have observed that NF-κB activation induced by proinflammatory cytokines is modulated by HSP90 inhibitors in vascular cells. In addition, it has been previously observed that NF-κB inhibition decreased inflammatory processes in the ApoE$^{-/-}$ model of atherosclerosis.[9,30] Other approaches, such as NF-κB decoy oligodeoxynucleotides have also been successfully applied in animal models of vascular disease.[31] In this respect, we have observed that HSP90 inhibitors decreased NF-κB activation, MCP-1 levels, and inflammatory cell infiltration in atherosclerotic plaques. These results are in agreement with previous papers in which HSP90 inhibitors attenuate NF-κB in other inflammatory diseases.[14,15]

Heat shock proteins are involved in protecting the tissue cells from a variety of insults. Although the mechanism of this protection has largely been thought to be due to their chaperone functions, it is known that some HSP, such as HSP70, displays anti-inflammatory properties in different diseases.[32] In this respect, HSP70 inhibits leucocyte adhesion and recruitment.[33] Moreover, mice overexpressing HSP70 showed decreased number of activated macrophages and inhibition of NF-κB in a model of brain inflammation.[34] In a model of colitis, Tanaka *et al.*[35] found that up-regulation of HSP70 in transgenic mice inhibited the expression of several cytokines, including IL6. In this respect, it has been observed the beneficial effect of HSP70 induction by 17-AAG treatment in experimental autoimmune encephalomyelitis by suppressing glial inflammatory responses.[13] Our results suggest that HSP70 induction by 17-AAG/DMAG could be an additional mechanism to decrease inflammation in vascular cells and atherosclerotic plaques.

The clinical significance of our results is related to the emerging evidence pointing out a role for HSP as potential therapeutic targets in different diseases.[25] In this respect, induction of HSP70 and degradation of client proteins by HSP90 inhibitors are able to decrease inflammation in different experimental models. However, since HSP90 inhibition affects several pathways at the same time, other pathological processes such as oxidative stress, neoangiogenesis, or apoptosis could also be affected by these drugs and would require further studies to clarify the protective role of HSP90 inhibitors in human diseases. In this respect, the dosing and toxicity of 17-AAG gained from Phase I clinical trials in cancer patients should facilitate the evaluation of HSP90 inhibitors in non-neoplastic disorders, such as cardiovascular diseases. For this purpose, 17-DMAG is a more potent and water soluble derivative than 17-AAG,[36,37] which can be administered orally, thus possibly making it a more feasible long-term therapeutic agent. Our results demonstrate that HSP90 inhibitors reduce inflammatory responses in atherosclerosis, suggesting that HSP90 could be a novel therapeutic target in atherosclerosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We want to thank Yolanda Vicente Lago for technical assistance, Carmen Gomez-Guerrero for helpful comments and Natalia Escribano for characterization of human plaques.

Conflict of interest: none declared.

Funding

This work was supported by the Spanish Ministerio de Ciencia y Tecnología (SAF2007/63648), Fundacion Ramon Areces, CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035), and European Network (HEALTH F2-2008-200647).

References

1. Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: global burden of disease study. *Lancet* 1997;**349**:1269–1276.
2. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2000;**20**:1262–1275.
3. Redgrave JN, Gallagher P, Lovett JK, Rothwell PM. Critical cap thickness and rupture in symptomatic carotid plaques: the oxford plaque study. *Stroke* 2008;**39**:1722–1729.
4. Martín-Ventura JL, Blanco-Colio LM, Muñoz-García B, Gómez-Hernández A, Arribas A, Ortega L *et al.* NF-kappaB activation and Fas ligand overexpression in blood and plaques of patients with carotid atherosclerosis: potential implication in plaque instability. *Stroke* 2004;**35**:458–463.
5. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med (Maywood)* 2003;**228**:111–133.
6. Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med* 2004;**82**:488–499.
7. Adhikari N, Charles N, Lehmann U, Hall JL. Transcription factor and kinase-mediated signaling in atherosclerosis and vascular injury. *Curr Atheroscler Rep* 2006;**8**:252–260.
8. Blanco-Colio LM, Tuñón J, Martin-Ventura JL, Egido J. Antiinflammatory and immunomodulatory effects of statins. *Kidney Int* 2003;**63**:12–23.
9. López-Franco O, Hernández-Vargas P, Ortiz-Muñoz G, Sanjuán G, Suzuki Y, Ortega L *et al.* Parthenolide modulates the NF-kappaB-mediated inflammatory responses in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006;**26**:1864–1870.
10. Ortiz-Muñoz G, Martin-Ventura JL, Hernandez-Vargas P, Mallavia B, Lopez-Parra V, Lopez-Franco O *et al.* Suppressors of cytokine signaling modulate JAK/STAT-mediated cell responses during atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009;**29**:525–531.
11. Malhotra V, Shanley TP, Pittet JF, Welch WJ, Wong HR. Geldanamycin inhibits NF-kappaB activation and interleukin-8 gene expression in cultured human respiratory epithelium. *Am J Respir Cell Mol Biol* 2001;**25**:92–97.
12. Wax S, Piecyk M, Maritim B, Anderson P. Geldanamycin inhibits the production of inflammatory cytokines in activated macrophages by reducing the stability and translation of cytokine transcripts. *Arthritis Rheum* 2003;**48**:541–550.
13. Dello Russo C, Polak PE, Mercado PR, Spagnolo A, Sharp A, Murphy P *et al.* The heat-shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin suppresses glial inflammatory responses and ameliorates experimental autoimmune encephalomyelitis. *J Neurochem* 2006;**99**:1351–1362.
14. Chatterjee A, Dimitropoulou C, Drakopanayiotakis F, Antonova G, Snead C, Cannon J *et al.* Heat shock protein 90 inhibitors prolong survival, attenuate inflammation, and reduce lung injury in murine sepsis. *Am J Respir Crit Care Med* 2007;**176**:667–675.
15. Poulaki V, Iliaki E, Mitsiades N, Mitsiades CS, Paulus YN, Bula DV *et al.* Inhibition of Hsp90 attenuates inflammation in endotoxin-induced uveitis. *FASEB J* 2007;**21**:2113–2123.
16. Moreno JA, Muñoz-García B, Martín-Ventura JL, Madrigal-Matute J, Orbe J, Páramo JA *et al.* The CD163-expressing macrophages recognize and internalize TWEAK. Potential consequences in atherosclerosis. *Atherosclerosis* 2009;**207**:103–110.
17. Martín-Ventura JL, Madrigal-Matute J, Muñoz-Garcia B, Blanco-Colio LM, Van Oostrom M, Zalba G *et al.* Increased CD74 expression in human atherosclerotic plaques: contribution to inflammatory responses in vascular cells. *Cardiovasc Res* 2009;**83**:586–594.
18. Wasserman BA, Wityk RJ, Trout HH 3rd, Virmani R. Response to Letter by Karapanayiotides et al. *Stroke* 2006;**37**:1647.
19. Lang SA, Klein D, Moser C, Gaumann A, Glockzin G, Dahlke MH *et al.* Inhibition of heat shock protein 90 impairs epidermal growth factor-mediated signaling in gastric cancer cells and reduces tumor growth and vascularization in vivo. *Mol Cancer Ther* 2007;**6**:1123–1132.
20. Hollingshead M, Alley M, Burger AM, Borgel S, Pacula-Cox C, Fiebig H *et al.* In vivo antitumor efficacy of 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride), a water-soluble geldanamycin derivative. *Cancer Chemother Pharmacol* 2005;**56**:115–125.
21. Sausville EA, Tomaszewski JE, Ivy P. Clinical development of 17-allylamino, 17-demethoxygeldanamycin. *Curr Cancer Drug Targets* 2003;**3**:377–383.
22. Kaartinen M, Penttilä A, Kovanen PT. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* 1994;**90**:1669–1678.
23. Martín-Ventura JL, Ortego M, Esbrit P, Hernández-Presa MA, Ortega L, Egido J. Possible role of parathyroid hormone-related protein as a proinflammatory cytokine in atherosclerosis. *Stroke* 2003;**34**:1783–1789.
24. Businaro R, Profumo E, Tagliani A, Buttari B, Leone S, D'Amati G *et al.* Heat-shock protein 90: a novel autoantigen in human carotid atherosclerosis. *Atherosclerosis* 2009;**207**:74–83.
25. Sőti C, Nagy E, Giricz Z, Vígh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. *Br J Pharmacol* 2005;**146**:769–780.
26. Rice JW, Veal JM, Fadden RP, Barabasz AF, Partridge JM, Barta TE *et al.* Small molecule inhibitors of Hsp90 potently affect inflammatory disease pathways and exhibit activity in models of rheumatoid arthritis. *Arthritis Rheum* 2008;**58**:3765–3775.
27. Seki Y, Kai H, Shibata R, Nagata T, Yasukawa H, Yoshimura A *et al.* Role of the JAK/STAT pathway in rat carotid artery remodeling after vascular injury. *Circ Res* 2000;**87**:12–18.
28. Gharavi NM, Alva JA, Mouillesseaux KP, Lai C, Yeh M, Yeung W *et al.* Role of the Jak/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids in vitro and in atherosclerosis in vivo. *J Biol Chem* 2007;**282**:31460–31468.

29. Recinos A 3rd, Lejeune WS, Sun H, Lee CY, Tieu BC, Lu M *et al.* Angiotensin II induces IL-6 expression and the Jak-STAT3 pathway in aortic adventitia of LDL receptor-deficient mice. *Atherosclerosis* 2007;**194**:125–133.
30. Cuaz-Perolin C, Billiet L, Baugé E, Copin C, Scott-Algara D, Genze F *et al.* Antiinflammatory and antiatherogenic effects of the NF-kappaB inhibitor acetyl-11-keto-beta-boswellic acid in LPS-challenged ApoE−/− mice. *Arterioscler Thromb Vasc Biol* 2008;**28**:272–277.
31. Yoshimura S, Morishita R, Hayashi K, Yamamoto K, Nakagami H, Kaneda Y *et al.* Inhibition of intimal hyperplasia after balloon injury in rat carotid artery model using cis-element 'decoy' of nuclear factor-kappaB binding site as a novel molecular strategy. *Gene Ther* 2001;**8**:1635–1642.
32. Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG. Antiapoptotic and Anti-inflammatory Mechanisms of Heat-Shock Protein Protection. *Ann N Y Acad Sci* 2005;**1053**:74–83.
33. House SD, Guidon PT Jr, Perdrizet GA, Rewinski M, Kyriakos R, Bockman RS *et al.* Effects of heat shock, stannous chloride, and gallium nitrate on the rat inflammatory response. *Cell Stress Chaperones* 2001;**6**:164–171.
34. Zheng Z, Kim JY, Ma H, Lee JE, Yenari MA. Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. *J Cereb Blood Flow Metab* 2008;**28**:53–63.
35. Tanaka K, Namba T, Arai Y, Fujimoto M, Adachi H, Sobue G *et al.* Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against colitis. *J Biol Chem* 2007;**282**:23240–23252.
36. Smith V, Sausville EA, Camalier RF, Fiebig HH, Burger AM. Comparison of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17DMAG) and 17-allylamino-17-demethoxygeldanamycin (17AAG) in vitro: effects on HSP90 and client proteins in melanoma models. *Cancer Chemother Pharmacol* 2005;**56**:126–137.
37. Herbst M, Wanker EE. Small molecule inducers of heat-shock response reduce polyQ-mediated huntingtin aggregation. A possible therapeutic strategy. *Neurodegener Dis* 2007;**4**:254–260.

3.2. La inhibición de HSP90, mediante el 17-DMAG, disminuye el estrés oxidativo en aterosclerosis experimental.

Para desarrollar el tercer objetivo de la tesis estudiamos el papel de la proteína HSP90 en el estrés oxidativo que subyace en el origen y desarrollo de la aterosclerosis, además del posible efecto antioxidante de su inhibición mediante el uso del 17-DMAG. La aterosclerosis se define como un proceso inmuno-inflamatorio que se origina entre otras causas por la acumulación subendotelial de LDLs, pudiendo ser oxidadas por ROS a LDLox. En las etapas tempranas de la aterosclerosis los macrófagos derivados de monocitos internalizan las LDLox a través de receptores basurero como CD36 [53] dando lugar a las células espumosas [194]. La migración y el acúmulo de células espumosas en la neoíntima, modulas por CD36 y las ROS producidas por la NADPH oxidasa, son factores claves en el desarrollo de la placa de ateroma [176]. De esta forma, la producción de ROS participa en el origen y desarrollo de la lesión aterosclerótica a través de diferentes mecanismos incluidos el estrés oxidativo y la inflamación. Hay numerosas proteínas cliente de HSP90 que participan en ambos procesos, como MEK y diversas subunidades de la NADPH oxidasa. Así, el uso del inhibidor de HSP90, 17-DMAG, disminuyó los niveles de ROS y la activación de ERK en las placas ateroscleróticas de un modelo de aterosclerosis experimental con ratones ApoE$^{-/-}$. El tratamiento *in vitro* de CMLVs de rata con el 17-DMAG disminuyó la producción de ROS y la actividad NADPH oxidasa inducida por TNF-α. Estos resultados están relacionados con los efectos pleiotrópicos de la inhibición de HSP90 por el 17-DMAG, como son el incremento en los niveles de HSP27 y HSP70, y la modulación de diversas rutas de señalización, como es la inhibición de ERK. Asimismo, realizamos estudios mecanísticos, mediante el uso de siRNAs específicos para HSP70 y HSP90, intentando diseccionar el efecto individualizado de las acciones duales del 17-DMAG. Los datos mostraron que la inhibición de HSP90 disminuyó de manera drástica la producción de ROS dependiente de la actividad de la NADPH oxidasa, mientras que el uso de un siRNA específico para HSP70 mostró el efecto totalmente opuesto. Estos datos están en consonancia con los resultados hallados con el 17-DMAG, ya que parece que tanto la inhibición de HSP90 como la sobreexpresión de HSP70 podrían estar colaborando en la modulación del estrés oxidativo. Además, en CMLVs la expresión de las subunidades de la NAPDH oxidasa, NOX1 y NOXO1, fue modulada por el 17-DMAG, tanto en la presencia como en la ausencia de TNF-α.

Por otro lado, usamos monocitos humanos para estudiar el efecto de la inhibición de HSP90 en otras células implicadas en la enfermedad vascular y analizar la acción del 17-DMAG en un tratamiento a largo plazo. Hallamos que el uso del 17-DMAG en monocitos humanos

(línea celular THP1) inhibió el proceso de diferenciación monocito/macrófago estimulado por PMA a 24 y 48h, comprobado mediante el análisis del marcador de diferenciación monocito-macrófago CD36. Además, los resultados muestran un incremento en las subunidades de la NAPDH oxidasa, NOX1 y NOXO1, asociadas al aumento de en la producción de ROS, a los niveles de expresión de CD36 y a las modificaciones fenotípicas producidas por el PMA en los THP-1. Todos estos procesos fueron revertidos mediante la inhibición de HSP90 por el 17-DMAG.

Finalmente, observamos una fuerte tinción tanto de NOX1 como de NOXO1 en la zona inflamatoria de las placas humanas de aterosclerosis avanzadas, colocalizando con CMLVs, macrófagos y células que producen altos niveles de ROS. Esta tinción fue confirmada mediante análisis por western blot de segmentos de aorta definidos como complicados y sus respectivos no complicados, mostrando los primeros unos mayores niveles proteicos de NOX1 y NOXO1. La zona complicada de la placa se define como la zona de la lesión, localizada generalmente en el origen interno de la carótida y que suele ser responsable de la cirugía. Contiene altos niveles de células inflamatorias (Stary stages V–VI), mientras que la zona fibrosa no complicada está compuesta principalmente por CMLVs y depósitos lipídicos (Stary stage III).

Todos estos datos muestran que el 17-DMAG tiene un efecto beneficioso en el desequilibrio redox presente en la pared vascular y podría promover la estabilidad de la placa a través de la modulación de factores prooxidativos y la inhibición de la transición monocito-macrófago.

Artículo publicado en *Cardiovascular Research*. 2012 Apr 30; Epub ahead to print.

Cardiovascular Research Advance Access published April 30, 2012

HSP90 INHIBITION BY 17-DMAG ATTENUATES OXIDATIVE STRESS IN EXPERIMENTAL ATHEROSCLEROSIS

Julio Madrigal-Matute, Carlos Ernesto Fernandez-Garcia, Carmen Gomez-Guerrero, Oscar Lopez-Franco, Begoña Muñoz-Garcia, Jesus Egido, Luis Miguel Blanco-Colio, Jose Luis Martin-Ventura.

Vascular Research Laboratory, IIS-Fundacion Jimenez Diaz, Autonoma University, Madrid, Spain.

Corresponding author:
Jose Luis Martin-Ventura, PhD
Vascular Research Lab.
IIS-Fundacion Jimenez Diaz
Autonoma University
Av. Reyes Católicos 2
28040. Madrid. Spain
Phone: +34915504800
Fax: +34915504399
jlmartin@fjd.es

ABSTRACT

Aims: Reactive oxygen species (ROS) participate in atherogenesis through different mechanisms including oxidative stress and inflammation. Proteins implicated in both processes, such as Mitogen-activated protein kinase kinase (MEK) and some NADPH oxidase (NOX) subunits are Heat shock protein-90 (HSP90) client proteins. In this work we investigated the antioxidant properties of the HSP90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) in experimental atherosclerosis.

Methods and results: Treatment of ApoE$^{-/-}$ mice with 17-DMAG (2 mg/kg every 2 days for 10 weeks) decreased ROS levels and extracellular-signal-regulated kinase (ERK) activation in aortic plaques compared to control animals. Accordingly, treatment of rat vascular smooth muscle cells (VSMCs) with 17-DMAG increased HSP27 and HSP70 and inhibited ERK activation. Interestingly, 17-DMAG diminished NADPH oxidase-ROS production in VSMCs and monocytes. Besides, a marked reduction in NADPH oxidase-ROS production was observed by HSP90siRNA and the opposite pattern with HSP70siRNA. 17-DMAG also diminished the expression of Nox1 and Nox organizer-1 (Noxo1) in VSMCs and monocytes. Interestingly, 17-DMAG was able to modulate ROS-induced monocyte to macrophage differentiation. Finally, higher expression of Nox1 and Noxo1 was found in the inflammatory region of human atherosclerotic plaques, colocalizing with VSMCs, macrophages and ROS producing cells.

Conclusion: Our results suggest that HSP90 inhibitors interfere with oxidative stress and modulate experimental atherosclerosis development through reduction of pro-oxidative factors.

INTRODUCTION

Atherosclerosis is defined as a chronic immune-inflammatory disease caused by sub-endothelial accumulation of non-native low-density lipoproteins (LDLs), which can be oxidised by reactive oxygen species (ROS) to oxidised LDLs (oxLDLs). In the early stages of atherosclerosis, monocyte-derived macrophages internalise oxLDLs through scavenger receptors such as Cluster of Differentiation 36 (CD36) [1] ultimately leading to foam cell formation [2]. Migration and deposition of foam cells in the neointima - modulated by CD36 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS production - are major contributors to plaque development [3]. Thus, two processes mediated by ROS, oxidative stress and inflammation [4, 5], interact to promote and aggravate atherosclerosis.

ROS are produced by different mechanisms, with NADPH oxidases being a major source of ROS in the cardiovascular system [6, 7]. Several pro-atherogenic factors such as phorbol-12-myristate-13-acetate (PMA) [8] or tumour necrosis factor alpha (TNFα) [9] regulate NADPH activity. Interestingly, a reduction of NADPH oxidase activity is associated with decreased atherosclerotic lesion size [10]. NADPH oxidases are membrane-associated multi-enzymes whose classical phagocytic structure is comprised of five subunits: the cytosolic factors p67phox, p47phox and p40phox; and the membrane-associated cytochrome b588, which contains the subunits gp91phox and p22phox [11]. Several homologues of the catalytic core gp91phox (Nox2) are found in non-phagocytic cells such as vascular cells [12, 13] and have been included in the so-called Nox family, which consists of seven homologues (Nox1-5 and Duox1-2). In large vessels, the predominant NADPH oxidases of vascular smooth muscle cells (VSMCs) are Nox1 [14] and Nox4 [15, 16], both contributing to ROS production. Nox4 is a constitutive enzyme with low catalytic activity, and is involved in physiological signalling. In

contrast, Nox1 is inactive in basal conditions [17], shows lower expression but high catalytic activity under pathological conditions, and is associated with cardiovascular damage [18, 19]. In VSMCs, Nox1 is complexed with p47phox and its homologue Nox organizer-1 (Noxo1). Noxo1 is constitutively active because it lacks the auto-inhibitory motif [20], while Nox activator-1 (Noxa1) is crucial for Nox1 activation and replaces p67phox in rodents [21].

Heat shock protein 90 (HSP90) is a ubiquitous molecular chaperone that ensures the proper conformation of different proteins, including key mediators of signal transduction and transcriptional regulation [22]. Selective HSP90 inhibitors, such as 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), block the ATP-binding site of HSP90 and exert pleiotropic functions which include induction of the heat shock response and degradation of some client proteins [23]. HSP90 inhibitors modulate different signalling pathways including mitogen-activated protein kinases (MAPKs) [24]. The MAPK member mitogen-activated protein kinase kinase (MEK), an HSP90 client protein, is in charge of extracellular-signal-regulated kinase 1/2 (ERK1/2) phosphorylation, a factor that upon activation mediates key processes of VSMCs in atherosclerosis [25]. We have recently reported that 17-DMAG diminishes the inflammatory response in atherosclerosis through upregulation of atheroprotective HSPs (e.g. HSP70) and inhibition of pro-inflammatory transcription factor activity and chemokine expression [26]. In the present study, we hypothesise that HSP90 inhibition reduces oxidative stress responses in experimental atherosclerosis.

METHODS

Reagents

The HSP90 inhibitor 17-DMAG (Biomol) was diluted in saline at 10 mM, stored at -20ºC and was used at a final concentration of 200 nM [26]. TNFα was purchased from Preprotech and used at 25 ng/mL [27]. The NADPH oxidase inhibitor apocynin was added to the cultures for 1 hour, at a final concentration of 100 µM [28]. PMA was used at 100 nM [29]. Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich.

Experimental Atherosclerosis

Twelve-week-old male ApoE$^{-/-}$ mice (Jackson Laboratory) were fed on a Western diet [21.2 % fat (0.5 % cholesterol) + 16.7 % protein] for eleven weeks. After one week of feeding, mice were randomised into two groups: 17-DMAG treatment (n=11) and control (n=9). The treated group was i.p. injected with 2 mg/kg of 17-DMAG every two days for ten weeks The control group consisted of ApoE$^{-/-}$ mice injected with vehicle (saline) as described [26]. The aorta was embedded in OCT and frozen for immunohistochemistry. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by our Institutional Committee for Animal Care and Use (IIS-Fundación Jiménez Díaz).

Cell culture

Male Wistar rats were euthanised with 200 mg/kg of pentobarbital sodium, their aorta was removed, and VSMCs were isolated and maintained in DMEM (BioWhittaker) supplemented with 10% foetal bovine serum (FBS, BioWhittaker), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Cells were used between passages 3 and 7. Human THP-1 monocytic cell line was purchased from

ATCC (CRL-1593) and cultured in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. For *in vitro* experiments in VSMCs, cells were made quiescent by incubation in medium without FBS for 24h.

Detection of superoxide anion

Dihydroethidium (DHE; Molecular Probes, Invitrogen) was used to evaluate *in situ* levels of superoxide as described previously [30] (see supplemental material online).

Transfection of small-interfering RNA

Rat VSMCs were grown to 20-50% confluence and transfected with scrambled (4611, Ambion), HSP70 (4390771, Ambion) or HSP90 α/β (sc-156099, Santa Cruz Biotechnologies) siRNAs using Lipofectamine™ RNAi MAX transfection reagent according to the instructions of the manufacturer (Invitrogen Life Technologies). The ability of the siRNA to inhibit HSP70 and HSP90 expression was assessed 48h post-transfection by real-time PCR and western-blot.

Patients

Ten atherosclerotic plaques (stages V-VI) from patients undergoing carotid endarterectomy in our institutions were fixed with paraformaldehyde and embedded in paraffin for histological analysis. In addition, ten carotid endarterectomy samples were dissected by a trained vascular surgeon separating the stenosing culprit plaque (CP) from the non-complicated (NCP) fibrous adjacent area as previously described [31]. The CP was defined as the lesion, usually localised at the origin of the internal carotid artery responsible for the surgery. The CP contained an important proportion of inflammatory cells (Stary stages V–VI), whereas the NCP adjacent areas were mainly composed of VSMCs and lipid deposits (Stary stage III). The study was approved by the hospital's ethics committee (IIS-Fundación Jiménez Díaz) according to the institutional and the

Good Clinical Practice guidelines, which was performed in accordance with the Declaration of Helsinki. All participants gave written informed consent.

Immunohistochemistry

OCT-embedded samples from mice arteries were incubated with rabbit polyclonal anti-pERK1/2 (sc-16981-R, Santa Cruz Biotechnologies), followed by rabbit secondary antibody. ABComplex/HRP (Vector laboratories) was added, sections were stained with 3,3'-diaminobenzidine (Dako) and mounted in Pertex (Histolab). Once immunohistochemistry had been carried out, data was collected using a Nikon Eclipse D400 microscope using bright field imaging at 20X magnification with NIKON ACT-1 software. Quantification of positive staining was performed using Image Pro Plus® The proven Solution™ version 4.5. Positive staining in the regions of interest was computed with the use of positive colour intensity thresholds. Quantification was performed by a pathologist, blind to the experimental groups analysed. Immunohistochemistry data are expressed as percentage of positive immunostained area vs. total area as described [30].

Paraffin-embedded human carotid atherosclerotic plaques were cross-sectioned into 4 µm-thick pieces, dewaxed, and rehydrated. For colocalisation studies in human plaques, double immunohistochemistry/immunofluorescence with rabbit polyclonal anti-Nox1 (sc-25545, Santa Cruz Biotechnologies) and Noxo1 (AP09301PU-N, Acris Antibodies GmbH) were carried out in the same sections followed by either α-actin (clone 1A4, M0851, Dako) or CD68 (clone kp1, M0814, Dako) staining, as previously described [32]. Following ABComplex/HRP for Nox1 or Noxo1, sections were stained with 3-amino-9-ethylcarbazole (AEC) plus High sensitivity chromogen (K3461, DAKO), followed by immunofluorescence for either α-actin or CD68 and mounted in Pertex. For DHE colocalisation in human plaques, Noxo1 and Nox1 antibodies were applied, ABComplex/HRP was added after secondary antibodies, sections were stained with

3,3'diaminobenzidine, followed by DHE staining and mounted in Pertex. Negative controls using the corresponding IgG were included for checking non-specific staining.

Western Blot

Equal amounts of protein lysates from human plaques and from the *in vitro* experiments were separated on 12.5% SDS-PAGE, transferred to PVDF membranes and then immunoblotted with antibodies against phospho-ERK1/2 (sc-7383, Santa Cruz Biotechnologies), ERK2 (recognizing both ERK1 and 2, sc-154, Santa Cruz Biotechnologies), Nox1 (sc-25545, Santa Cruz Biotechnologies), Noxo1 (AP09301PU-N, Acris Antibodies GmbH), HSP90 (sc-13119), HSP70 (SPA-810, Stressgen), HSP27 (sc-1049, Santa Cruz Biotechnologies) and β-actin (sc-47778, Santa Cruz Biotechnologies). Proteins were visualised by ECL Western Blotting Detection Reagents (Amersham Biosciences) according to manufacturer's instructions.

RNA extraction and Real-Time Quantitative-Polymerase Chain Reaction

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen). One µg of RNA was used to perform the reverse transcription with High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR reactions were performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to the manufacturer's protocol, using the ΔΔCt method as described [32]. Rat mRNA levels for HSP90α, HSP90β, HSP70, HSP27, Nox1, Nox4 and p22phox and human mRNA levels for CD36 were done by amplification of cDNA using SYBR® Premix Ex Taq™ (Takara Biotechnology). The primer sequences are listed in Supplemental Table 1 (online). The mRNA levels of NADPH oxidase subunits and HSPs were normalised to GAPDH mRNA content. Pre-developed primers and probe assays were obtained for eukaryotic 18S, rat and human Noxo1, human Nox1 and rat Noxa1 from Applied Biosystems.

Expression levels are given as ratio to the housekeeping gene 18S and data obtained after treatment is expressed as fold change when compared to basal values.

Measurement of NADPH activity

The lucigenin-enhanced chemiluminescence assay was used to determine the NADPH oxidase activity in cell homogenates as previously described [33]. Briefly, cells were lysed in buffer containing protease inhibitors (1‰ protease inhibitor cocktail) and subjected to Dounce homogenisation (100 times, on ice), and the homogenate was stored until use. The reaction mixture comprised 50 mM phosphate buffer, 0.01 mM EDTA, 0.32 M sucrose, 5 µM lucigenin and 0.1 mM NADPH, in a total volume of 500 µL. A low concentration of lucigenin (5 µM) was used to avoid auto-oxidation. The reaction was started by the addition of lucigenin to the cell homogenate (50 µg of protein) and the light emission was recorded every 10 seconds for 5 min in a tube luminometer Sirius (Berthold Detection System). There was no measurable activity in the absence of NADPH. Data obtained after treatment is expressed as fold change relative to untreated control.

Measurement of NADPH-dependent ROS production

Lucigenin-enhanced chemiluminescence assay [27] was used to determine the NADPH-dependent ROS production activity in fresh monocytes and rat VSMCs. The reaction mixture comprised 50 mM phosphate buffer containing 1mM EGTA, pH 7.0, 5 µM lucigenin and 0.1 mM NADPH. The chemiluminescence, which was measured for 5 minutes after the addition of NADPH, was recorded in a luminometer Sirius (Berthold Detection System). No activity could be measured in the absence of NADPH. The ROS production was determined from the ratio of relative light units to total protein levels and expressed as fold vs. basal.

Statistical analysis

In vitro experiments were performed at least three times. Results are expressed as mean ± S.E.M. and were analysed by the Mann–Whitney non-parametric or Student´s t-test when appropriate. Wilcoxon paired test was used to analyse the differences between NCP and CP from the same patients. Univariate association between HSP70 and DHE staining in mice atherosclerotic plaques was performed by Spearman correlation test. Statistics were performed using GraphPAD InStat (GraphPAD Software). Differences were considered significant with $p < 0.05$.

RESULTS

HSP90 inhibition by 17-DMAG diminishes ROS production and ERK activation *in vivo*

We previously showed that *in vivo* treatment with 17-DMAG attenuated lesion size, lipid content and macrophage infiltration in atherosclerotic plaques of ApoE$^{-/-}$ mice [26]. Using the same animal model, we found a significant decrease in superoxide levels in the 17-DMAG group compared to the vehicle-injected group (Figure 1A and B). Since we previously observed that HSP70 was increased in the 17-DMAG injected group [26], we analysed the possible relationship between DHE staining and HSP70 expression in the atherosclerotic plaques. Analysis of serial sections from mice aortic plaques revealed a significant negative correlation between DHE staining and HSP70 expression ($r = -0.5$; $p < 0.001$).

Furthermore, since ERK activation is involved in ROS production, and the upstream kinase MEK is an HSP90 client protein, we studied the effect of 17-DMAG treatment on ERK phosphorylation in mice atherosclerotic plaques. We found decreased ERK 1/2 phosphorylation in the 17-DMAG-treated group compared to vehicle-injected animals (Figure 1C and D).

Pleiotropic effects of 17-DMAG in VSMCs

The effects of HSP90 inhibition by 17-DMAG include the induction of some members of the HSP family and the modulation of several signalling pathways. We assessed HSP27 and HSP70 expression under basal and pro-oxidative conditions. We found an increase - at the protein and mRNA levels - in HSP27 and HSP70 induced by 17-DMAG but not by TNFα treatment (Supplemental Figure 1). No cytotoxic effects of 17-DMAG were observed at the doses and times studied (data not shown).

Next, we examined the effect of this HSP90 inhibitor on ERK1/2 activation. We show that treatment with 17-DMAG diminishes basal ERK1/2 phosphorylation at 4 and 8h (Figure 2A and B). To further evaluate the effects of 17-DMAG on ERK1/2 under a pro-oxidative scenario, we stimulated VSMCs with TNFα. This pro-oxidative cytokine rapidly increased ERK1/2 phosphorylation, an effect abolished by HSP90 inhibitor pre-treatment (Figure 2C and D).

17-DMAG decreases ROS production and NADPH oxidase activity in VSMCs

To analyse the mechanisms involved in the modulation of oxidative stress by 17-DMAG *in vivo,* cultured VSMCs were incubated with TNFα in the absence or presence of 17-DMAG. Exposure to TNFα augmented ROS levels in VSMCs, which was prevented by 17-DMAG pre-treatment, while 17-DMAG alone did not significantly modify basal ROS levels (Figure 3A and B). The potential involvement of NADPH oxidase in the ROS production by VSMCs was confirmed by the use of the inhibitor apocynin, which abolished the pro-oxidative effect of TNFα (Figure 3B). Furthermore, 17-DMAG pre-treatment inhibited the NADPH oxidase activity induced by TNFα in VSMCs (Figure 3C). Interestingly, 17-DMAG was also able to decrease the basal levels of NADPH oxidase activity (Figure 3C). To study the specific effect of HSP90 inhibition, we measured basal NADPH-dependent ROS production under genetic

silencing of either HSP90 or HSP70 (Supplemental Figure 2). We found that genetic inhibition of HSP90 abolished ROS production while HSP70 silencing resulted in a marked increase (Figure 3D).

17-DMAG attenuates NADPH oxidase subunit expression

HSP90 is a chaperone that interacts with multiple client proteins, among them some NADPH subunits [34]. Thus, we examined the expression of major NADPH subunits in VSMCs such as Nox1, Noxo1, p22phox, Nox4 and Noxa1 and the potential modulation by 17-DMAG. In basal conditions, HSP90 inhibition by 17-DMAG treatment diminished Nox1 and Noxo1 mRNA levels (Figure 4A). Furthermore, incubation of cells with TNFα for 4h gave rise to an increase in the catalytic NADPH oxidase subunit Nox1 and the regulatory subunit Noxo1 at both the protein and mRNA level (Figure 4B and C). The induction of Nox1 and Noxo1 expression was prevented by pre-treatment with 17-DMAG (Figure 4B and C). In contrast, no significant effects of TNFα were observed on Nox4, p22phox and Noxa1 mRNA expression at the dose and times studied (Figure 4D).

17-DMAG prevents NADPH oxidase-dependent ROS production and macrophage differentiation *in vitro*

To study the effects of HSP90 inhibition on other relevant cells present in the atherosclerotic plaque, parallel studies were performed in monocytes (THP-1 human cell line). PMA induced NADPH-dependent ROS production (Figure 5A) and Nox1 and Noxo1 expression (Figure 5B) in a time-dependent manner in THP-1 cells, which was significantly reduced by 17-DMAG pre-treatment. Similar to VSMCs, basal NADPH oxidase-dependent ROS generation and Nox1 and Noxo1 levels were also reduced by 17-DMAG in monocytes. Interestingly, 17-DMAG prevented the cell morphology changes associated with monocyte-to-macrophage differentiation by PMA in our

experimental conditions (Figure 5C). This was further verified by analysing CD36 expression, a macrophage differentiation marker. PMA time-dependently induced CD36 mRNA, which was diminished by 17-DMAG (Figure 5D).

Nox1 and Noxo1 are increased in the culprit plaques of human atherosclerotic plaques

To elucidate the potential relevance of our studies in human atherosclerosis we examined the expression of Nox1 and Noxo1 proteins in human carotid atherosclerotic plaques. We found that Nox1 and Noxo1 staining was mainly localised in the inflammatory region of the shoulder, characterised by a high macrophage accumulation, although it was also present in the fibrous region, with increased VSMCs and collagen content (Figure 6A and B). Colocalisation studies confirmed that both Nox1 and Noxo1 are expressed by CD68 and α-actin positive cells in advanced human carotid atherosclerotic plaques (Supplemental Figure 3A and B). In addition, we found colocalisation of Noxo1 and Nox1 with DHE positive cells (Supplemental Figure 3C). Lastly, we quantified Nox1 and Noxo1 levels by Western blotting in tissue extracts from complicated areas (CP, containing an important proportion of inflammatory cells, Stary stages V–VI) and the adjacent non-complicated region (NCP, mainly composed of VSMCs and lipid deposits, Stary stage III) of the plaques. Western blot analysis revealed a significant increase in both NADPH subunits in the CP region of human advanced atherosclerotic plaques as compared to the NCP areas (Figure 6C and D).

DISCUSSION

Since ROS are a common feature of the 'vicious circle' of oxidative stress and inflammation in the arterial wall, therapies to prevent the pathological augmentation of ROS production in the vascular system are of interest [35]. We found that treatment with the HSP90 inhibitor 17-DMAG significantly reduced ROS levels in atherosclerotic plaques of ApoE$^{-/-}$ mice when compared to controls. Moreover, *in vitro* experiments

showed analogous results in VSMCs and monocyte-derived macrophages, as 17-DMAG partially reversed the TNFα- or PMA-induced ROS production.

The pleiotropic effects of HSP90 inhibition by 17-DMAG include induction of HSP synthesis and degradation of HSP90 client proteins, thereby modulating several signalling pathways. In one hand, we show that pre-treatment with the HSP90 inhibitor 17-DMAG upregulates HSP27 and HSP70 mRNA and protein levels in VSMCs. Interestingly, we found an inverse correlation between HSP70 expression and DHE staining in the atherosclerotic plaques from ApoE$^{-/-}$ mice. Accordingly, we found that HSP70 knockdown markedly increased ROS generation in VSMCs. In this context, previous studies have shown that overexpression of HSP70 exerts anti-oxidative effects in a rat model of atherosclerosis [36]. Furthermore, the anti-oxidant effects of resveratrol treatment in atherosclerosis are accompanied by a dose-dependent augmentation of HSP27 [37]. On the other hand, the pleiotropic effects of HSP90 inhibition include modulation of several signalling pathways such as the MAPK cascade, in particular ERK1/2, a well-known redox-sensitive kinase. Accordingly, mice treated with 17-DMAG showed decreased levels of ERK1/2 activation in atherosclerotic plaques. This effect was corroborated by *in vitro* experiments in which stimulation of VSMCs with 17-DMAG was able to reverse both basal and TNFα-induced ERK1/2 phosphorylation. In agreement, previous studies showed that HSP90 inhibition using 17-AAG [24] or 17-DMAG [38] diminishes ERK phosphorylation in different cell types. Thus, upregulation of atheroprotective HSPs and attenuation of ERK activation by 17-DMAG could inhibit oxidative stress in atherosclerosis.

Interestingly, 17-DMAG was also able to reduce both basal and TNFα-induced NADPH oxidase activity, a main source of ROS in vascular cells. Since the NADPH catalytic subunit Nox1 and HSP90 interact through the C-terminal residue of Nox1, it is

tempting to speculate that 17-DMAG/HSP90 binding prevents Nox1/HSP90 interaction [34]. In this respect, we have observed that HSP90 inhibition by HSP90α/β siRNA clearly inhibited basal NADPH-dependent ROS production in VSMCs. Moreover, we found that 17-DMAG was able to reduce both the basal and the TNFα-induced expression of Nox1 and Noxo1, but not the mRNA levels of other NADPH subunits analysed. In agreement with Chen *et al.*, the reduced levels in Nox subunits following HSP90 inhibition is not necessarily related to NADPH oxidase activity reduction, but it is another effect of HSP90 inhibition that could be atheroprotective during long-term treatment [34]. In the present work, we demonstrate that 17-DMAG can effectively inhibit PMA-induced NADPH oxidase-dependent ROS production, Nox1 and Noxo1 overexpression, together with CD36, a hallmark of monocyte-to-macrophage differentiation. Thus, the long term functional consequences of HSP90 inhibition by 17-DMAG could include decreasing NADPH-dependent ROS production involved in macrophage differentiation, a key process implicated in foam cell retention and atherogenesis [3].

To clarify the relevance of Nox1 and Noxo1 modulation in atherosclerosis, we analysed the expression of both proteins in human carotid atherosclerotic plaques. Although Nox1 mRNA levels have been previously analysed in human atherosclerotic plaques [39], we are not aware of any studies that have addressed Nox1 or Noxo1 protein levels in human atherosclerotic plaques. We first performed an observational study in which we found that Nox1 and Noxo1 staining was mainly localised in the inflammatory region of the shoulder, characterised by a high macrophage accumulation, although it was also present in the fibrous region. Moreover, we show Nox1 and Noxo1 expression not only in macrophages but also in VSMCs of human atherosclerotic plaques. Besides, we found colocalisation between both subunits and DHE positive

cells, which suggests that cells expressing high levels of Nox1 and Noxo1 produce elevated levels of ROS. We further quantified and confirmed an increased protein expression of Nox1 and Noxo1 in the culprit region of the human atherosclerotic plaques compared to their respective non-complicated region. This data highlights the potential importance of Nox1-derived ROS in the chronic immune-inflammatory oxidative scenario present in human advanced atherosclerotic lesions.

In summary, we have shown that 17-DMAG is able to reduce oxidative stress in experimental atherosclerosis. Although further studies are needed to clarify the role of HSP90 as a possible therapeutic target in atherosclerosis, its present use in clinical trials [40] should facilitate the evaluation of HSP90 inhibitors in non-neoplastic disorders, such as cardiovascular diseases.

FUNDING

This work was supported by the Spanish Ministerio de Ciencia y Tecnología (SAF 2010-21852), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (PI10/00072), Sociedad Española de Aterosclerosis and Lilly.

ACKNOWLEDGMENTS

We wish to thank Patricia Llamas Granada for technical assistance and Laura Madrigal-Esteban for editing this article.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem* 1993;**268**:11811-11816.

2. Rahaman SO, Lennon DJ, Febbraio M, Podrez EA, Hazen SL, Silverstein RL. A CD36-dependent signaling cascade is necessary for macrophage foam cell formation. *Cell Metab* 2006;**4**:211-221.

3. Park YM, Febbraio M, Silverstein RL. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *J Clin Invest* 2009;**119**:136-145.

4. Albertini R, Moratti R, De LG. Oxidation of low-density lipoprotein in atherosclerosis from basic biochemistry to clinical studies. *Curr Mol Med* 2002;**2**:579-592.

5. Lavrovsky Y, Chatterjee B, Clark RA, Roy AK. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp Gerontol* 2000;**35**:521-532.

6. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK *et al*. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996;**97**:1916-1923.

7. Mohazzab KM, Wolin MS. Sites of superoxide anion production detected by lucigenin in calf pulmonary artery smooth muscle. *Am J Physiol* 1994;**267**:L815-L822.

8. Fortuno A, Olivan S, Beloqui O, San JG, Moreno MU, Diez J *et al*. Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension. *J Hypertens* 2004;**22**:2169-2175.

9. Lassegue B, Griendling KK. NADPH oxidases: functions and pathologies in the vasculature. *Arterioscler Thromb Vasc Biol* 2010;**30**:653-661.

10. Fenyo IM, Florea IC, Raicu M, Manea A. Tyrphostin AG490 reduces NAPDH oxidase activity and expression in the aorta of hypercholesterolemic apolipoprotein E-deficient mice. *Vascul Pharmacol* 2011;**54**:100-106.

11. Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. *Arch Biochem Biophys* 2002;**397**:342-344.

12. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J *et al*. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation* 2004;**109**:227-233.

13. Manea A, Raicu M, Simionescu M. Expression of functionally phagocyte-type NAD(P)H oxidase in pericytes: effect of angiotensin II and high glucose. *Biol Cell* 2005;**97**:723-734.

14. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y *et al*. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001;**88**:888-894.

15. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004;**24**:677-683.

16. Ellmark SH, Dusting GJ, Fui MN, Guzzo-Pernell N, Drummond GR. The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc Res* 2005;**65**:495-504.

17. Geiszt M, Lekstrom K, Witta J, Leto TL. Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *J Biol Chem* 2003;**278**:20006-20012.

18. Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HH, Harrison DG, Griendling KK. Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free Radic Biol Med* 2008;**45**:1340-1351.

19. Dikalova A, Clempus R, Lassegue B, Cheng G, McCoy J, Dikalov S *et al*. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. *Circulation* 2005;**112**:2668-2676.

20. Cheng G, Lambeth JD. NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *J Biol Chem* 2004;**279**:4737-4742.

21. Ambasta RK, Schreiber JG, Janiszewski M, Busse R, Brandes RP. Noxa1 is a central component of the smooth muscle NADPH oxidase in mice. *Free Radic Biol Med* 2006;**41**:193-201.

22. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med (Maywood)* 2003;**228**:111-133.

23. Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med* 2004;**82**:488-499.

24. Hostein I, Robertson D, DiStefano F, Workman P, Clarke PA. Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. *Cancer Res* 2001;**61**:4003-4009.

25. Blanc A, Pandey NR, Srivastava AK. Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H2O2 in vascular smooth muscle cells: potential involvement in vascular disease (review). *Int J Mol Med* 2003;**11**:229-234.

26. Madrigal-Matute J, Lopez-Franco O, Blanco-Colio LM, Munoz-Garcia B, Ramos-Mozo P, Ortega L *et al*. Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis. *Cardiovasc Res* 2010;**86**:330-337.

27. Manea A, Tanase LI, Raicu M, Simionescu M. Transcriptional regulation of NADPH oxidase isoforms, Nox1 and Nox4, by nuclear factor-kappaB in human aortic smooth muscle cells. *Biochem Biophys Res Commun* 2010;**396**:901-907.

28. Zalba G, Fortuno A, Orbe J, San JG, Moreno MU, Belzunce M *et al*. Phagocytic NADPH oxidase-dependent superoxide production stimulates matrix metalloproteinase-9: implications for human atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007;**27**:587-593.

29. Moreno JA, Munoz-Garcia B, Martin-Ventura JL, Madrigal-Matute J, Orbe J, Paramo JA *et al*. The CD163-expressing macrophages recognize and internalize TWEAK: potential consequences in atherosclerosis. *Atherosclerosis* 2009;**207**:103-110.

30. Lopez-Franco O, Hernandez-Vargas P, Ortiz-Munoz G, Sanjuan G, Suzuki Y, Ortega L *et al*. Parthenolide modulates the NF-kappaB-mediated inflammatory responses in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006;**26**:1864-1870.

31. Martin-Ventura JL, Leclercq A, Blanco-Colio LM, Egido J, Rossignol P, Meilhac O *et al*. Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. *Atherosclerosis* 2007;**194**:334-341.

32. Martin-Ventura JL, Ortego M, Esbrit P, Hernandez-Presa MA, Ortega L, Egido J. Possible role of parathyroid hormone-related protein as a proinflammatory cytokine in atherosclerosis. *Stroke* 2003;**34**:1783-1789.

33. Manea A, Manea SA, Gafencu AV, Raicu M, Simionescu M. AP-1-dependent transcriptional regulation of NADPH oxidase in human aortic smooth muscle cells: role of p22phox subunit. *Arterioscler Thromb Vasc Biol* 2008;**28**:878-885.

34. Chen F, Pandey D, Chadli A, Chen T, Catravas JD, Fulton D. Hsp90 regulates NADPH oxidase activity and is necessary for superoxide but not hydrogen peroxide production. *Antioxid Redox Signal* 2011.

35. Levonen AL, Vahakangas E, Koponen JK, Yla-Herttuala S. Antioxidant gene therapy for cardiovascular disease: current status and future perspectives. *Circulation* 2008;**117**:2142-2150.

36. Okada M, Hasebe N, Aizawa Y, Izawa K, Kawabe J, Kikuchi K. Thermal treatment attenuates neointimal thickening with enhanced expression of heat-shock protein 72 and suppression of oxidative stress. *Circulation* 2004;**109**:1763-1768.

37. Wang Z, Chen Y, Labinskyy N, Hsieh TC, Ungvari Z, Wu JM. Regulation of proliferation and gene expression in cultured human aortic smooth muscle cells by resveratrol and standardized grape extracts. *Biochem Biophys Res Commun* 2006;**346**:367-376.

38. Hackl C, Mori A, Moser C, Lang SA, Dayoub R, Weiss TS *et al*. Effect of heat-shock protein-90 (HSP90) inhibition on human hepatocytes and on liver regeneration in experimental models. *Surgery* 2010;**147**:704-712.

39. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP *et al*. Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 2002;**105**:1429-1435.

40. Biamonte MA, Van de Water R, Arndt JW, Scannevin RH, Perret D, Lee WC. Heat shock protein 90: inhibitors in clinical trials. *J Med Chem* 2010;**53**:3-17.

FIGURE LEGENDS

Figure 1. Effects of 17-DMAG on ROS levels and ERK activation in atherosclerotic plaques from ApoE$^{-/-}$ mice. (A) Representative DHE staining in aortic samples from control and 17-DMAG-treated mice (magnification x100). (B) Quantification of ROS production in the lesions. (C) Representative images of ERK1/2 activation in control and 17-DMAG-injected mice (magnification x50). (D) Quantification of pERK1/2 positive staining in aortic plaques of ApoE$^{-/-}$ mice. Values shown are means ± SEM, control (n=9) and 17-DMAG (n=11). #p < *0.05* vs. control.

Figure 2. Effect of HSP90 inhibition on ERK activation in rat VSMCs. (A) Representative immunoblots and (B) quantification of the ratio phosphorylated/total ERK1/2 in VSMCs treated with 17-DMAG (200 nM, at the indicated times). (C) Representative immunoblot and (D) quantification of ERK1/2 activation in VSMCs treated with TNFα (25 ng/mL, 30 min) in the presence or absence of 17-DMAG (200 nM, 4h pre-incubation). Values shown are means ± SEM of three independent experiments. #p < *0.05* vs. basal and *p < *0.05* vs. TNFα.

Figure 3. ROS production in rat VSMCs. (A) Representative DHE staining of rat VSMCs under basal conditions and stimulated with TNFα (25 ng/mL, 10 min) in the presence or absence of 17-DMAG (200 nM, 4h pre-incubation). (B) Quantification of ROS production by DHE staining in rat VSMCs [TNFα (25 ng/mL, 10 min), 17-DMAG (200 nM, 4h pre-incubation), Apocynin (100 µM, 1h pre-incubation)]. (C) NADPH oxidase activity in rat VSMCs stimulated with or without TNFα (25 ng/mL, 10 min), and in the absence or presence of 17-DMAG (200 nM, 4h pre-incubation). (D)

NADPH-dependent ROS production in rat VSMCs transfected with vehicle (-), scrambled (scr) and specific siRNA for HSP90α/β (si90) and HSP70 (si70) (30 nM, 24h for all). Values shown are means ± SEM of three independent experiments. #p < *0.05* vs. basal and *p < *0.05* vs. TNFα.

Figure 4. Effect of HSP90 inhibition on NADPH subunit expression in rat VSMCs. (A) Nox1 and Noxo1 mRNA quantification by real-time PCR in VSMCs treated with 17-DMAG (200 nM, at the indicated times). (B) Representative immunoblots of Nox1, Noxo1 and β-actin protein in VSMCs treated with TNFα (25 ng/mL, 4h) in the absence or presence of 17-DMAG (200 nM, 4h pre-incubation). (C) Nox1 and Noxo1 mRNA quantification by real-time PCR in VSMCs treated with TNFα (25 ng/mL, 4h) in the absence or presence of 17-DMAG (200 nM, 4h pre-incubation). (D) mRNA quantification of different NADPH oxidase subunits by real-time PCR of VSMCs treated with TNFα (25 ng/mL, 4h) in the absence or presence of 17-DMAG (200 nM, 4h pre-incubation). Values shown are means ± SEM of three independent experiments. #p < *0.05* vs. basal and *p < *0.05* vs. TNFα.

Figure 5. Effect of 17-DMAG on ROS production by THP-1 cells and in monocyte-to-macrophage differentiation. (A) NADPH-dependent ROS production in THP-1 cells treated with PMA (100 nM, 24 or 48h) in the absence or presence of 17-DMAG (200 nM, 4h pre-incubation). (B) Nox1 and Noxo1 mRNA quantification by real-time PCR in THP-1 treated with PMA (100 nM, 24 or 48h) in the absence or presence of 17-DMAG (200 nM, 4h pre-incubation). (C) Representative images of THP-1 differentiation induced by PMA (100 nM) in the absence or in the presence of 17-DMAG (200 nM, 4h pre-incubation) [(a) Basal, (b) PMA 24h, (c) PMA 48h, (d) 17-

DMAG, (e) PMA 24h + 17-DMAG and (f) PMA 48h + 17-DMAG (magnification X250)]. (D) CD36 mRNA quantification by real-time PCR in THP-1 cells treated with PMA (100 nM, 24 or 48h) in the absence or presence of 17-DMAG (200 nM, 4h pre-incubation). Values shown are means ± SEM of three independent experiments. #p < *0.05* vs. basal and *p < *0.05* vs. PMA. DM = 17-DMAG; P24 or P48 = PMA 24h or PMA 48h.

Figure 6. Nox1 and Noxo1 protein levels in human carotid atherosclerotic plaques. Representative images of (A) Nox1 and (B) Noxo1 in the inflammatory and fibrous regions of the plaques. Magnification x10 (detail x25). (C) Representative immunoblots and (D) quantification of Nox1, Noxo1 and GAPDH protein in non-complicated plaques (NCP) and their respective complicated plaques (CP). Values shown are means ± SEM, n= 10 per group. #p < *0.05* vs. NCP.

FIGURE 1

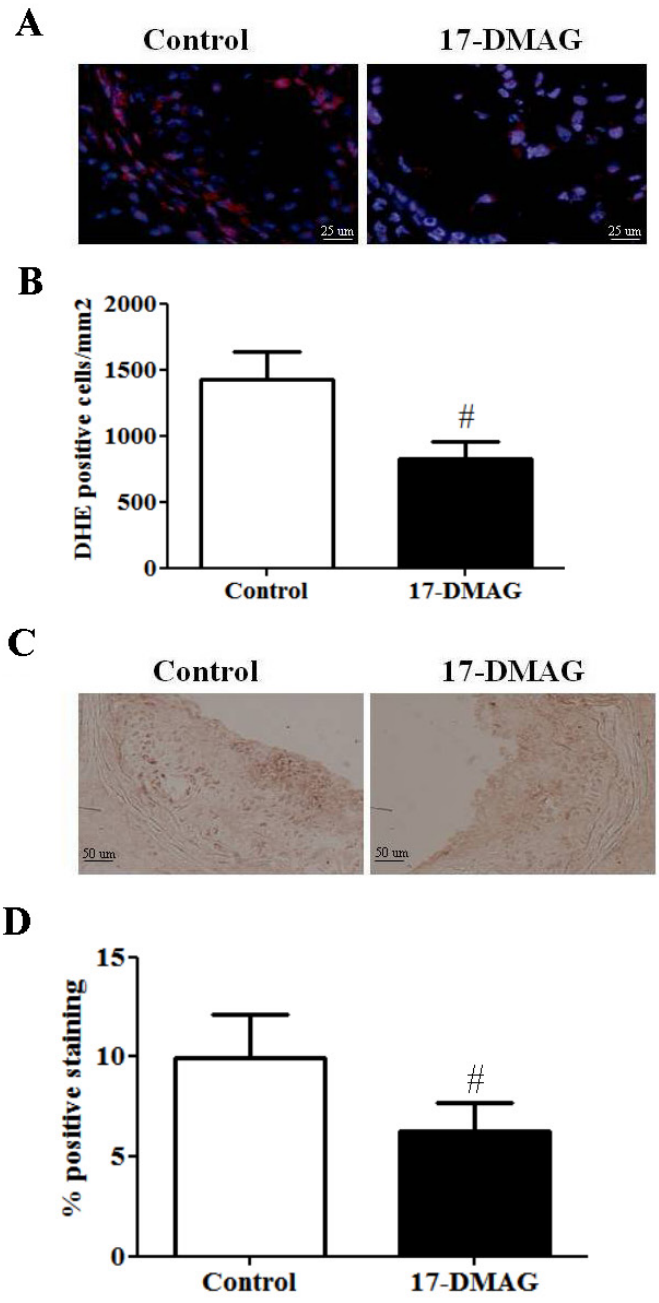


FIGURE 2

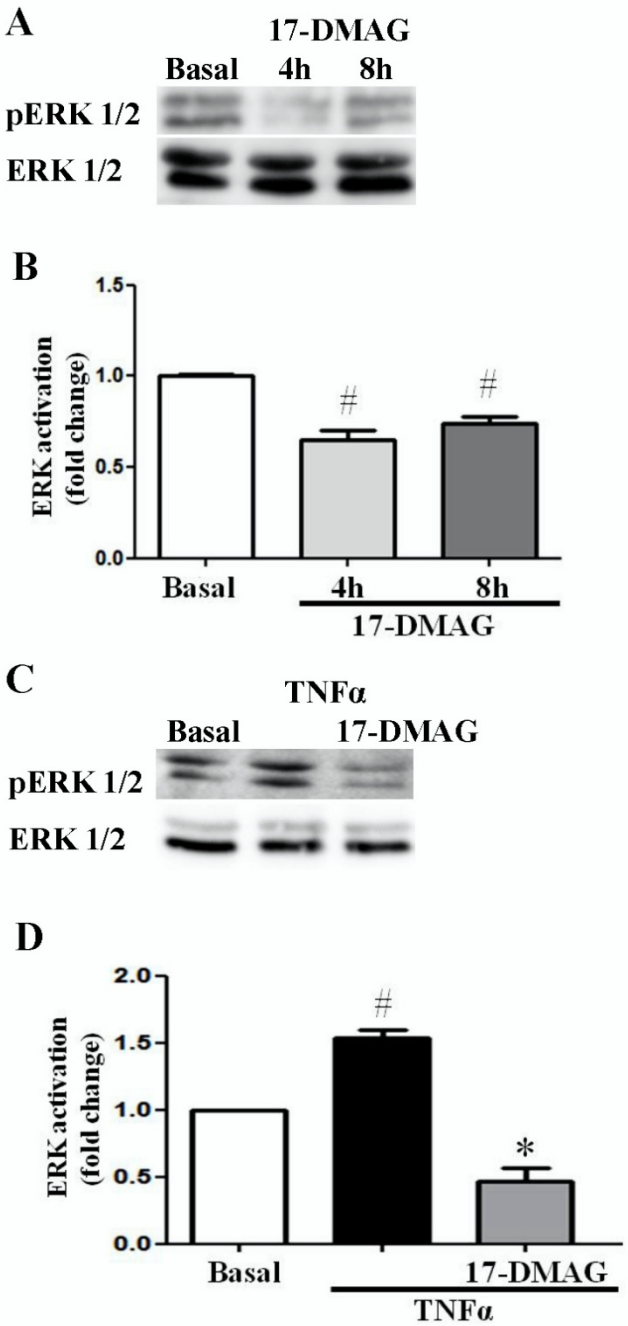

A
17-DMAG
Basal
4h
8h
pERK 1/2
ERK 1/2
B
ERK activation (fold change)
1.5
1.0
0.5
0.0
#
#
Basal
4h
8h
17-DMAG
C
TNFα
Basal
17-DMAG
pERK 1/2
ERK 1/2
D
ERK activation (fold change)
2.0
1.5
1.0
0.5
0.0
#
*
Basal
17-DMAG
TNFα

FIGURE 3

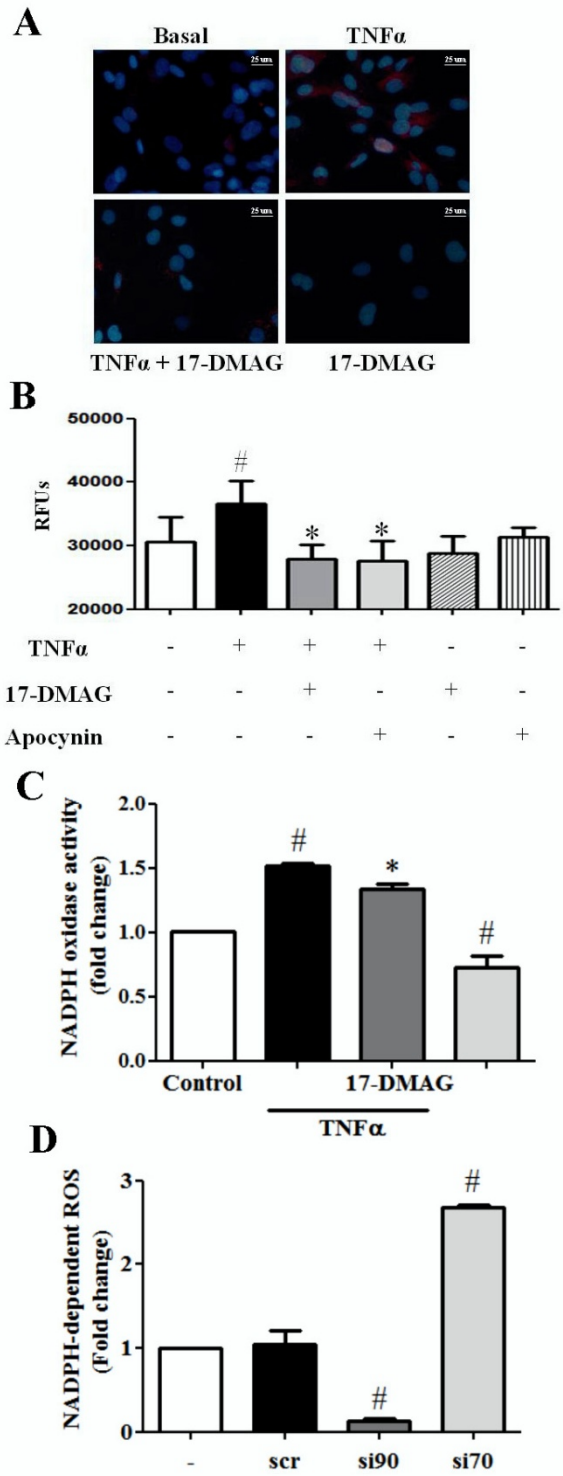

A
Basal
TNFα
TNFα + 17-DMAG
17-DMAG
B
RFUs
50000
40000
30000
20000
TNFα
17-DMAG
Apocynin
C
NADPH oxidase activity
(fold change)
2.0
1.5
1.0
0.5
0.0
Control
17-DMAG
TNFα
D
NADPH-dependent ROS
(Fold change)
3
2
1
0
-
scr
si90
si70

FIGURE 4

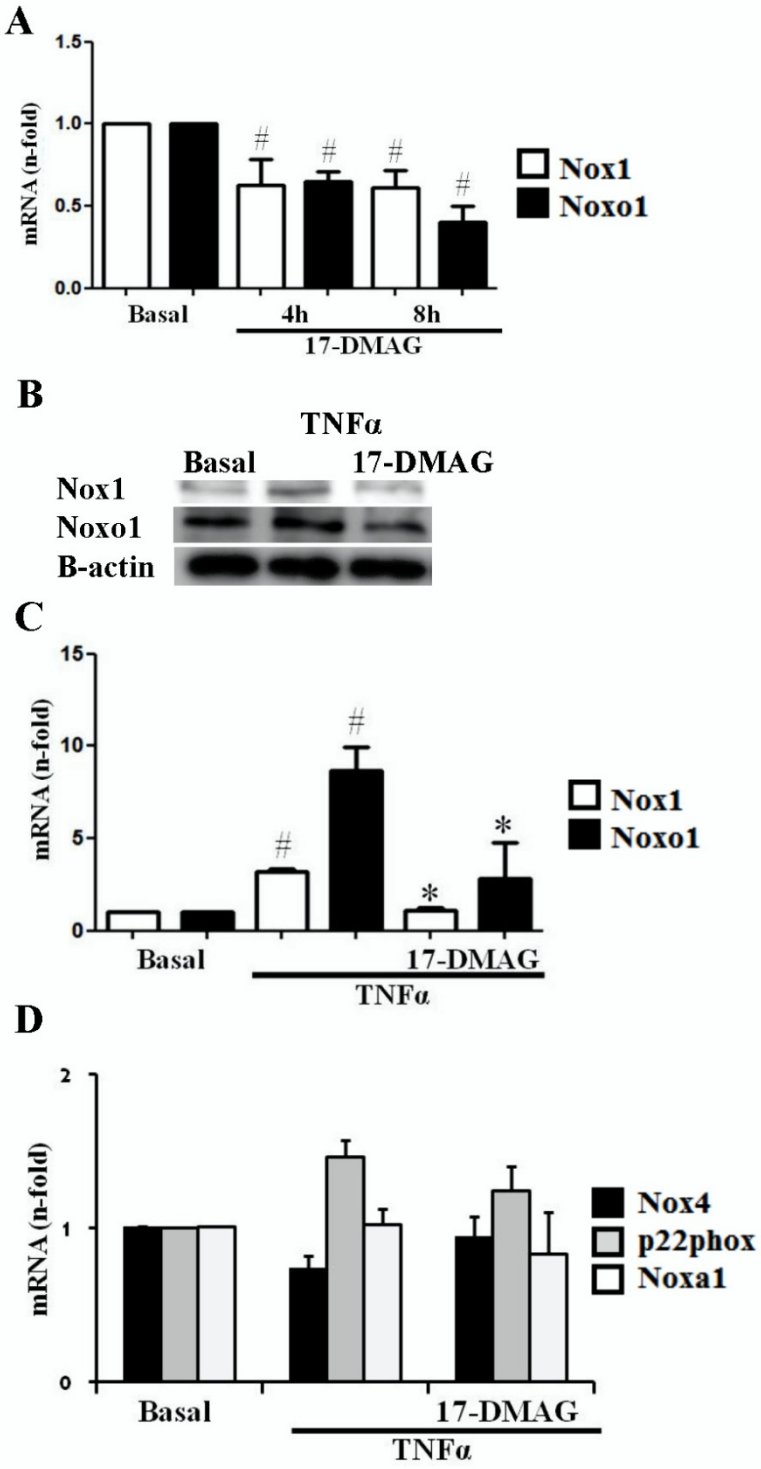

A
mRNA (n-fold)
1.5
1.0
0.5
0.0
#
#
#
#
Nox1
Noxo1
Basal
4h
8h
17-DMAG
B
TNFα
Basal
17-DMAG
Nox1
Noxo1
B-actin
C
mRNA (n-fold)
15
10
5
0
#
#
*
*
Nox1
Noxo1
Basal
17-DMAG
TNFα
D
mRNA (n-fold)
2
1
0
Nox4
p22phox
Noxa1
Basal
17-DMAG
TNFα

FIGURE 5

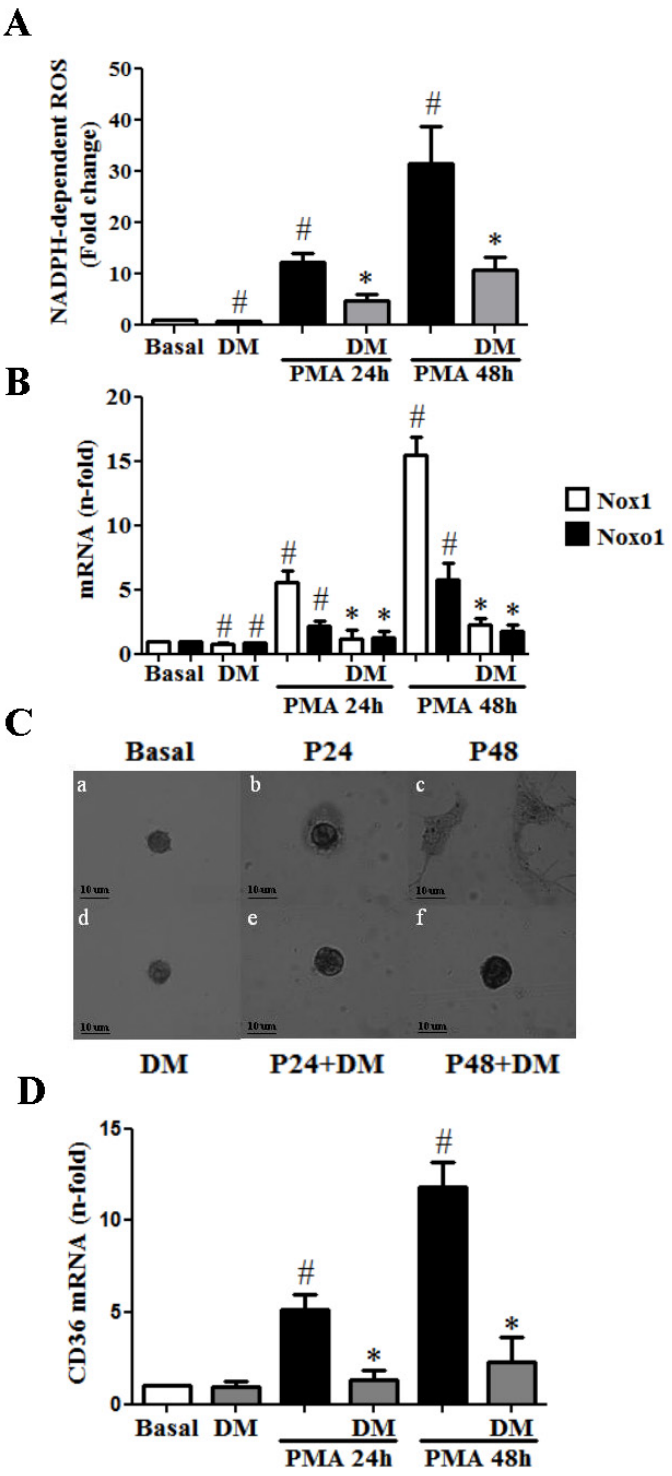
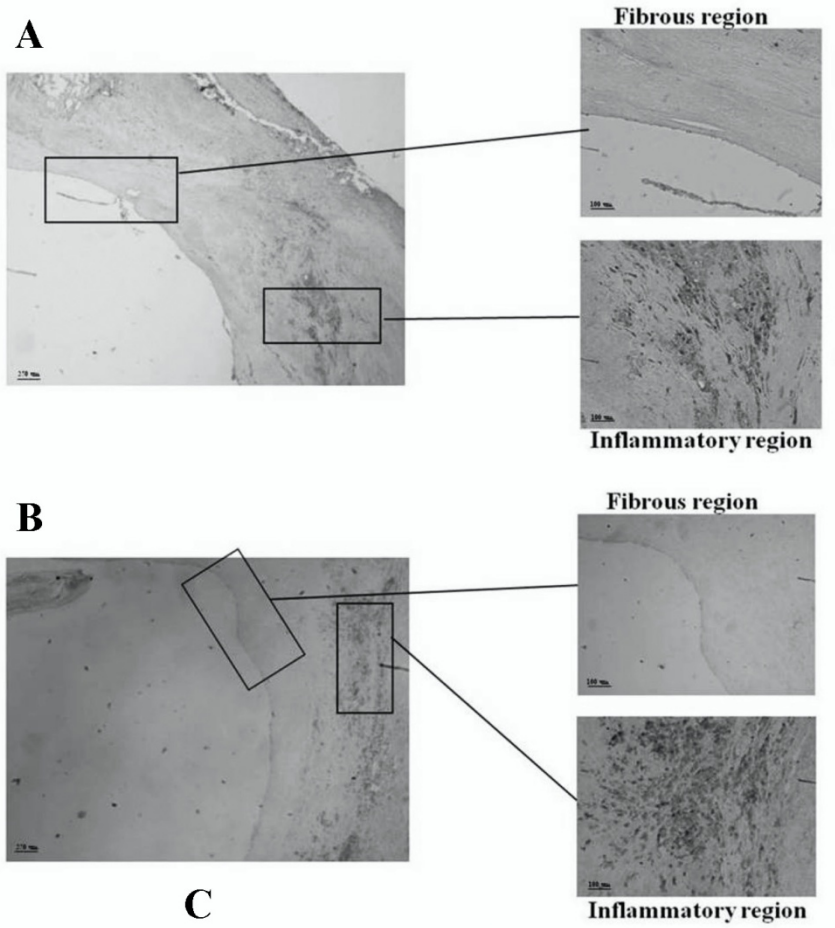

A
NADPH-dependent ROS (Fold change)
50
40
30
20
10
0
Basal
DM
DM
DM
PMA 24h
PMA 48h
B
mRNA (n-fold)
20
15
10
5
0
Nox1
Noxo1
C
Basal
P24
P48
a
b
c
d
e
f
DM
P24+DM
P48+DM
D
CD36 mRNA (n-fold)
15
10
5
0

FIGURE 6


A
Fibrous region
Inflammatory region
B
Fibrous region
Inflammatory region
C
NCP CP NCP CP
Nox1
Noxo1
GAPDH
D
A.U.
30
20
10
0
#
#
NCP CP NCP CP
Nox1
Noxo1

3.3. Los niveles de Peroxirredoxina-1/Tiorredoxina se asocian con el espesor de la íntima-media y la actividad NADPH oxidasa en sujetos con aterosclerosis subclínica.

Los objetivos cuarto y quinto de esta tesis se centraron en el estudio de otra familia de CSPs, como es el sistema de la TRX. Para desarrollar estos dos objetivos analizamos los niveles plasmáticos de PRDX-1/TRX, tanto en pacientes con estenosis carotídea como en sujetos asintomáticos con un grosor de la íntima-media conocido y la relación de las proteínas PRDX-1/TRX con la NAPDH oxidasa. Dentro de los diferentes sistemas antioxidantes, la TRX junto con la TRX reductasa y la NADPH oxidasa, constituyen un sistema ubicuo que regula el estado redox celular [266]. Las propiedades de la TRX en el reciclaje de las moléculas pro-oxidativas son ejercidas a través de las PRDX. Además, la PRDX puede interaccionar y modular la actividad NADPH oxidasa mediante la inactivación del H_2O_2 y su ruta de señalización [120]. Intracelularmente, aparte de su papel como antioxidantes, TRX y PRDX participan en numerosos procesos que van desde la síntesis de DNA, a la regulación de la apoptosis o la proliferación celular [188, 263]. En respuesta a diferentes tipos de estrés, tanto PRDX como TRX pueden ser secretadas al medio extracelular [208, 200]. En nuestro estudio, encontramos que los pacientes con aterosclerosis carotídea muestran mayores niveles de PRDX-1/TRX que los sujetos sanos. Además, en una población de 84 sujetos asintomáticos, encontramos una correlación positiva entre PRDX-1/TRX y el espesor íntima-media, ajustado por edad y sexo. Finalmente, mediante análisis de regresión hallamos que PRDX-1/TRX correlacionaron positivamente entre sí. Igualmente, analizamos la posible asociación entre PRDX-1/TRX y la actividad NADPH oxidasa, ya que PRDX-1/TRX correlacionaron positivamente con la producción de superóxido dependiente de la NADPH oxidasa en la población de pacientes asintomáticos. Así, para intentar ahondar en la potencial asociación entre PRDX-1/TRX y la NADPH oxidasa, usamos monocitos humanos en los que la estimulación con PMA incrementó los niveles extracelulares de PRDX-1/TRX y la actividad NADPH oxidasa, junto con una disminución en los niveles intracelulares de PRDX-1/TRX. Este efecto fue parcialmente revertido mediante el pretratamiento con apocinina, un inhibidor de la NADPH oxidasa. Sin embargo, para intentar demostrar la especificidad de la NADPH oxidasa en el proceso de liberación de PRDX/TRX realizamos experimentos de pérdida de función mediante el uso de un siRNA específico para NOX2, lo que bloqueó la disminución intracelular de PRDX-1/TRX inducida por PMA en macrófagos de ratón. Asimismo, en placas humanas de aterosclerosis avanzada, mostramos la colocalización de PRDX-1/TRX con CD68 (marcador de actividad

fagocítica, generalmente expresado por macrófagos), α-actina (marcador de CMLVs) y p22phox (subunidad reguladora de la NADPH oxidasa).

Como conclusión, hallamos que los niveles de PRDX-1/TRX están incrementados en pacientes con estenosis carotídea y correlacionan positivamente con el grosor íntima-media y la producción de superóxido dependiente de la actividad NADPH oxidasa en sujetos asintomáticos. Además, describimos una potencial implicación de la producción de ROS a través de la NADPH oxidasa en el proceso de secreción de PRDX/TRX al medio extracelular. Todos estos datos sugieren una respuesta coordinada antioxidante frente al incremento del estrés oxidativo en aterotrombosis.

Artículo en revisión en *Arteriosclerosis, Thrombosis, and Vascular Biology*.

PEROXIREDOXIN-1/THIOREDOXIN LEVELS ARE ASSOCIATED TO INTIMA MEDIA THICKNESS AND NADPH OXIDASE ACTIVITY IN SUBJECTS WITH SUBCLINICAL ATHEROSCLEROSIS.

Julio Madrigal-Matute[1], Luis Miguel Blanco-Colio[1], Ana Fortuño[2], Roxana Martinez-Pinna[1], Priscila Ramos-Mozo[1], Oscar Beloqui[3], Jesus Egido[1], Guillermo Zalba[2]*, José Luis Martin-Ventura[1]*.

*both seniors contribute equally to this work

[1] Vascular Research Lab, ISS-Fundación Jimenez Diaz, Autonoma University, Madrid, [2]Division of Cardiovascular Sciences, Center for Applied Medical Research University of Navarra, Pamplona. [3]Department of Internal Medicine, University Clinic, University of Navarra, Pamplona.

Total word count: 4910

5 Figures, 1 table, 3 tables online

Brief title: PRX-1, TRX AND SUBCLINICAL ATHEROSCLEROSIS

Address for correspondence:

Jose Luis Martin-Ventura, PhD

Vascular Research lab- IIS-Fundacion Jimenez Diaz- Autonoma University

Av. Reyes Catolicos 2

28040. Madrid. Spain

jlmartin@fjd.es

Telephone number: 0034 5504800 (Ext.3168)

Fax Number: 0034 915448246

ABSTRACT

Objectives: To analyze peroxiredoxin-1 (PRX-1) and thioredoxin (TRX) plasma levels in patients with carotid stenosis and in asymptomatic subjects with known intima-media thickness (IMT). To assess the potential association between PRX-1/TRX and NADPH oxidase (Nox) activity.

Methods and results: Patients with carotid atherosclerosis showed higher PRX-1 and TRX plasma levels than healthy subjects [37(21-66) vs 13(8-28) and 47(38-71) vs 17(15-30) ng/ml, respectively, $p<0.001$ for both]. In a test population of 84 asymptomatic subjects, we found a positive correlation between PRX-1/TRX and IMT ($r=0.31$ and 0.36; $p<0.01$ for both) adjusted by age and sex. PRX-1/TRX positively correlated with phagocytic NADPH oxidase-dependent superoxide production ($r=0.48$ and 0.47, $p<0.001$ for both). Finally, PRX-1 and TRX showed a highly significant positive correlation between them ($r=0.88$, $p<0.001$). In human atherosclerotic plaques, p22phox colocalized with PRX-1/TRX. Moreover, in human monocytes stimulated with PMA, increased PRX-1/TRX extracellular levels and NADPH oxidase activity were observed, with a concomitant decrease in PRX-1/TRX intracellular levels. In loss-of-function experiments, Nox2 silencing blocked PMA-induced intracellular PRX-1/TRX downregulation in macrophages.

Conclusions: PRX-1/TRX plasma levels are increased in patients with carotid stenosis and correlates with IMT and NADPH oxidase-dependent superoxide production in asymptomatic subjects. These data could suggest a coordinated antioxidant response to increased oxidative stress in atherothrombosis.

KEYWORDS: OXIDATIVE STRESS-BIOMARKERS-ATHEROSCLEROSIS-NADPH OXIDASE

Atherothrombosis is the leading cause of mortality in the Western world. The underlying pathological process is a thickening of the arterial wall due to the formation of atheromatous plaques. Oxidative stress is involved in both, the early stages (e.g. modificating/interacting with key molecules such as LDL or NO), as well as in the more advanced phases (e.g. stimulating the secretion of proteases that contribute to the weakening of the fibrous cap) of atherothrombosis (1,2).

Oxidative stress is the result of imbalance between antioxidant and prooxidant molecules. Among different antioxidants, thioredoxin (TRX) together with TRX reductase and nicotinamideadenindinucleotidephosphate (NADPH) constitutes a ubiquitous system that regulates cellular redox status (3). The oxidant scavenging properties of TRX are performed through TRX peroxidases or peroxiredoxins (PRX). Moreover, PRX can interact and modulate NADPH oxidase activity by inactivating H_20_2 and H_20_2-signalling pathways (4). Intracellularly, apart from their role as antioxidants, TRX and PRX participate in a multitude of fundamental processes, ranging from DNA synthesis to regulation of apoptosis and cell proliferation (5,6). In addition, TRX and PRX could be released to the extracellular environment (7,8). Extracellular TRX is present in the circulation and its levels increase under oxidative stress/inflammatory conditions (9). TRX is elevated in plasma of patients with atherothrombosis (10,11). In contrast, less is known regarding circulating PRX levels. In this respect, we have recently observed increased peroxiredoxin-1 (PRX-1) serum levels in patients with abdominal aortic aneurysm (AAA)(12). Moreover, PRX-1 levels positively correlated with AAA size and growth, suggesting its potential role as a diagnostic and prognostic biomarker of AAA.

On the other hand, reactive oxygen species (ROS) have different biological actions in the vasculature, mainly oxidative damage. ROS can also influence signalling pathways

through changes in the activity of redox-sensitive kinases (e.g. MAPK), transcription factors (e.g. NF-kB) or molecules (e.g. TRX)(13). NADPH oxidase system is one of the main sources of ROS in the vessel wall, and is present in endothelial cells, smooth muscle cells, fibroblasts and infiltrated monocytes/macrophages (14). Several works have demonstrated a key role of vascular and phagocytic NADPH oxidase (Nox) isoforms in the development of human vascular diseases (14). In this respect, we have previously demonstrated that NADPH oxidase activity positively correlated with carotid intima-media thickness (IMT) in asymptomatic subjects (15).

Although TRX and NADPH oxidase, together with PRX, constitute a key system in cardiovascular homeostasis, the relationship between them has not been completely defined so far. We therefore hypothesized that an association may exist between phagocytic NADPH oxidase and PRX-1/TRX in human atherosclerosis. In order to test this hypothesis, we have examined: (a) the relationship of plasma PRX-1/TRX with NADPH oxidase activity and subclinical atherosclerosis (IMT) in asymptomatic subjects, (b) the association between PRX-1/TRX and NADPH oxidase in carotid atherosclerotic plaques, and (c) the ability of NADPH oxidase to regulate PRX-1/TRX in cultured monocytes and macrophages.

METHODS

PATIENTS

Carotid atherosclerosis

Thirty consecutive patients undergoing carotid endarterectomy in our Institution were included in the study (69±8 years, 28 males, 73% hypertensive, 53% hyperlipidemic, 27% diabetic, 40% current smokers). Atherosclerotic plaques (Stary stages V-VI) were fixed with paraformaldehyde and embedded in paraffin. The local committees on human research at IIS-Fundacion Jimenez Diaz-Autonoma University approved the study, which was performed in accordance with the principles outlined in the Declaration of Helsinki, and all participants gave written informed consent.

Thirty controls were recruited from a screening program that is currently being performed among the population in the area under our care. They were randomly selected from the screened individuals with no stenosis in the carotid artery.

Asymptomatic subjects

In addition, a group of 84 asymptomatic subjects in whom global risk assessment was performed in the course of a general health check-up by Internal Medicine Department (University Clinic of Navarra, Spain) were also studied. In all subjects, absence of history of coronary disease, stroke or peripheral arterial disease was recorded. Additional exclusion criteria were the presence of severely impaired renal function, arthritis, connective tissue diseases, alcohol abuse, or use of nonsteroidal, anti-inflammatory drugs in the 2 weeks before entering the study. The following conventional cardiovascular risk factors were defined: arterial hypertension and/or use of anti-hypertensive drugs; dyslipidemia and/or use of cholesterol lowering drugs, obesity, smoking, and diabetes and/or use of pharmacological treatment. In all subjects, carotid ultrasonography was performed to determine IMT (16). Subjects were

examined by the same 2 sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination. The between-observer intraclass correlation coefficient was 0.76 ($P<0.001$) and the between subject repeatability was 0.82 ($P<0.001$). The corresponding coefficients of variance were 5% and 10%, respectively.

Cell culture

Human THP-1 monocytic cell line was purchased from ATCC (CRL-1593) and cultured with RPMI 1640 (BioWhittaker) supplemented with 10% decomplemented FBS, 2mM L-glutamine and antibiotics. For experiments, cells were preincubated with 0% FBS during 24h.

The mouse macrophagic cell line RAW 264.7 was obtained from the ATCC (TIB-71). Cells were cultured in DMEM supplemented with 10% FBS, 4 mM glutamine, 1 mM sodium piruvate and antibiotics.

Determination of superoxide production in blood cells

400,000 PBMCs isolated from blood samples with Lymphoprep were used to measured NADPH oxidase-dependent superoxide production in basal conditions and in response to stimulation with 3.2 μM phorbolmyristate acetate (PMA) (Sigma Aldrich), during 1 hour and by using 5 μM lucigenin (Sigma Aldrich). Luminescence measurements (1 s) were recorded every 15-30 s along an interval of 1 hour in a plate reader luminometer (Luminoskan Ascent, Labsystem) (17). The value of the area under the curve was used to quantify chemiluminescence. A buffer blank was subtracted from each reading. We have previously reported that lucigenin measurements closely correlated with an independent measurement of superoxide production using superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction.

Determination of NADPH oxidase activity in cultured cells

Chemiluminescence assays were used to measure superoxide production in THP-1 and RAW 264.7 cells. Cells were grown in medium with 10% FBS. After reaching 80-90% confluence, cells were maintained in medium without FBS and stimulated with PMA for different times (30min in THP-1 and 24h in RAW). Cells were then scrapped and homogeneized on ice. Protein concentration was measured by using the Lowry method. Chemiluminescence assays with lucigenin 5 μM and NADPH 0.1 mM were used to measure NADPH oxidase activity in 15 μg of protein homogenates. The reaction was started by addition of lucigenin to protein homogenates in a final volume of 300 μL. Luminescence was measured 5 minutes in a tube luminometer Sirius (Berthold Detection System). A buffer blank was substracted from each reading. The value of the area under the curve was used to quantify chemiluminescence. Data are expressed as relative light units produced per second.

siRNA experiments

Duplex siRNA specific for murine Nox2 (sense, 5'-GAUGCCUGGAAACUACCUAtt-3'; antisense, 5'-UAGGUAGUUUCCAGGCAUCtt-3') were synthesized (Ambion). Transfection of siRNA was performed by using Lipofectamine-RNAiMAX as previously (18). Experiments were also performed with a universal negative control (Sense sequence 5-UACCGUUGUUAUAGGUGUCtt-3') available from Ambion.

Western blot

Equal amount of total protein (volume for conditioned media or concentration for intracellular proteins) was separated on 12.5% sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). Subsequently, membranes were blocked and incubated with anti-thioredoxin antibody (Mouse SC-58440 clone 3A1), PRX-1

(Santa Cruz Biotechnology, goat polyclonal sc-23969), mouse monoclonal anti-α-tubulin (T-5168, Sigma-Aldrich), mouse monoclonal anti-Nox-2 (611414 clone 53, BD Transduction Laboratories) and mouse monoclonal anti-b-actin (A5441 clone AC15, Sigma-Aldrich) washed with TBS-T, and incubated with anti-mouse horseradish peroxidase antibody. Proteins were visualized by ECL Western Blotting Detection Reagents (Amersham Bioscienes) according to manufacturer's instructions.

ELISA

We quantified with commercial kits the soluble concentrations of TRX (Redox Bioscience) and PRX-1 (ABFRONTIER) in plasma, following manufacturer´s instructions. The interassay and intra-assay variabilities for TRX were 15% and 6% and for PRX-1 were 9% and 6%, respectively.

Immunohistochemistry/immunofluorescence

Paraffin-embedded human carotid atherosclerotic plaques were cross-sectioned into 4 µm-thick pieces, dewaxed, and rehydrated. For colocalization of PRX-1 (ab15571, abcam), TRX (sc-58440), CD68 (clone kp1, M0814) and p22phox (sc-20781) immunohistochemistry followed by immunofluorescence was performed as previously described (19). Negative controls using the corresponding IgG were included for checking non-specific staining.

Statistical analysis

Statistics were performed using SPSS 11.0. In vitro experiments were performed at least three times. Results are expressed as mean ± S.E.M. and were analyzed by the Mann–Whitney non-parametric, Wilcoxon paired or Student´s t-test when appropriate (two-tailed, significant differences at $p<0.05$).

PRX-1 and TRX levels in carotid atherosclerotic patients are expressed as median (interquartile ranges) and were analyzed by the Mann-Whitney U test. Data from the

asymptomatic population are expressed as mean±S.E.M. Univariate association was performed by Pearson correlation test. Multivariate linear regression analysis was conducted with carotid IMT, PRX-1 or TRX as dependent variables, including in the model the traditional risk factors and those variables that were significant in the univariate analysis.

RESULTS

PRX-1/TRX plasma levels in human carotid atherosclerotic patients

We analyzed PRX-1 plasma levels in a training group of 30 patients with carotid atherosclerosis and 30 healthy controls. PRX-1 levels in carotid atherosclerotic patients were higher than in control subjects [37(21-66) vs 13(8-28) ng/ml, $p<0.001$, Figure 1A]. In addition, although circulating TRX levels are increased in cardiovascular pathologies (10,11), no data regarding plasma TRX levels in carotid atherosclerotic patients is available. TRX levels in carotid atherosclerotic patients were higher than in control subjects [47(38-71) vs 17(15-30) ng/ml, $p<0.001$, Figure 1B]. Finally, PRX-1 and TRX showed a highly significant positive correlation between them ($r=0.8$, $p<0.01$).

PRX-1/TRX plasma levels in human asymptomatic subjects

We analyzed PRX-1 and TRX levels in a test population of 84 asymptomatic subjects in which carotid IMT had been measured. Characteristics of the studied population are summarized in Table 1 online. A positive correlation between PRX-1/TRX and carotid IMT was observed after adjustment for age and sex ($r=0.31$ and 0.36, respectively; $p<0.01$ for both) (Table 1). No correlation between PRX-1/TRX and other clinical parameters was observed (Table 1). In a multivariate analysis, the association between plasma PRX-1/TRX levels and carotid IMT remained statistically significant after adjusting for some potentially confounding cardiovascular risk factors (Table 2 online). Interestingly, PRX-1/TRX positively correlated with phagocytic NADPH oxidase-dependent superoxide production ($r=0.48$ and 0.47 respectively, $p<0.001$ for both) (Figure 2). In a multivariate analysis, the association between PRX-1/TRX levels and phagocytic NADPH oxidase-dependent superoxide production remained statistically significant after adjusting for some potential factors that might be regulating PRX-

1/TRX levels (Table 3 online). Finally, PRX-1 and TRX showed a highly significant positive correlation between them ($r=0.88$, $p<0.001$), reinforcing their potential value as biomarkers of oxidative stress.

PRX-1/TRX expression colocalizes with NADPH oxidase in atherosclerotic plaques

Although PRX-1 has been previously analyzed in atherosclerotic plaques in mice (20), no studies have addressed this issue in human atherosclerotic plaques. We have shown that PRX-1 in expressed in macrophages (Figure 3A), but also vascular smooth muscle cells and red blood cells (not shown), of advanced human carotid atherosclerotic plaques. In addition, PRX-1 was colocalized with TRX in atheroma plaques (Figure 3B). Following our hypothesis, we have studied the potential association of NADPH oxidase and PRX-1/TRX in human atherosclerotic plaques. We have shown that p22phox colocalized with PRX-1 (Figure 3C) and TRX (Figure 3D) in human atherosclerotic plaques.

PRX-1/TRX levels in monocytes/macrophages under oxidative stress

Since PMA is able to induce ROS production via NADPH oxidase activation, we further studied the potential association of PRX-1/TRX with NADPH oxidase by testing the effect of PMA on PRX-1/TRX levels in human monocytes. PRX-1/TRX intracellular levels were decreased in PMA-stimulated monocytes, with a concomitant increase in the conditioned media. This effect was reversed by pretreatment with apocynin, an inhibitor of NADPH oxidase (Figure 4). As expected, in these experimental conditions, NADPH oxidase activity was increased by PMA, which was attenuated by apocynin (1.7 ± 0.2 vs 1.3 ± 0.2 fold vs basal, $p<0.05$).

To demonstrate the specificity of NADPH oxidase on the PMA-induced PRX-1/TRX intracellular downregulation, we performed loss-of-function experiments using

siRNAs against the Nox2 subunit of NADPH oxidase in murine macrophages. Nox2 was effectively knocked down by Nox2-specific siRNA, as shown by Western blot (Figure 5A). Consequently, the silencing of Nox2 in Raw264.7 cells resulted in a significant decrease in NADPH oxidase activity in response to stimulation with PMA (Figure 5B). In control conditions (scramble siRNA), PRX-1/TRX intracellular levels were decreased in PMA-stimulated macrophages (Figure 5C), as previously shown in human monocytes. In contrast, siRNA of Nox2 blocked PMA-induced intracellular PRX-1/TRX downregulation (Figure 5C).

DISCUSSION

TRX and PRX-1 are overexpressed in response to oxidative stress as a mechanism to protect cells against oxidant injury (9,21). In this respect, circulating TRX is elevated in patients with oxidative stress-associated acute and chronic clinical conditions such as myocardial infarction and unstable angina (10,11). In two recent studies, we have described both PRX-1 and TRX as novel circulating biomarkers of AAA (12,22). Moreover, both PRX-1 and TRX serum levels correlated with AAA diameter and AAA growth. In the present paper, we have observed an increase in both PRX-1 and TRX plasma levels in patients with carotid atherosclerosis in relation to healthy subjects. Since increases in the thickness of the intima and media of the carotid artery (a surrogate marker of atherosclerosis) are directly associated with an increased risk of AMI and stroke (23), we evaluated the potential association of both PRX-1 and TRX with IMT in asymptomatic subjects. We have shown that PRX-1 and TRX plasma levels correlate with IMT, which remained significant after adjusting for classical risk factors. Interestingly, we have previously demonstrated that phagocytic NADPH oxidase activity correlates with carotid IMT in asymptomatic subjects free of overt clinical atherosclerotic disease (15). In the present paper, PRX-1 and TRX plasma levels positively correlates with NADPH oxidase-dependent superoxide production of PBMC in asymptomatic subjects. In agreement, circulating PRX-1 and TRX levels have been previously associated to other markers of oxidative stress (e.g. myeloperoxidase, 8-OHdG) in different pathologies (13, 24). Finally, PRX-1 and TRX show a high significant correlation in plasma of both carotid atherosclerotic patients and asymptomatic subjects, which could suggest a coordinated response of both proteins to increased oxidative stress present in atherothrombosis.

Increased expression levels of PRX-1 in aortas from apoE$^{-/-}$ mice with advanced atherosclerosis have been observed (20). In the present paper, we extend this data to human carotid atherosclerotic plaques, showing PRX-1 expression associated to macrophages, but also vascular smooth muscle cells and red blood cells. In this respect, it has been recently described that TRX is expressed in human atherosclerotic plaques associated to intraplaque hemorraghe, possibly as an antioxidant response of vascular cells to a prooxidant environment (25). Similarly, several members of the phagocytic NADPH oxidase have been detected in human atherosclerotic plaques (14), among them p22phox isoform of NADPH oxidase (26). In the present paper, we have shown that PRX-1/TRX colocalized with p22phox in human carotid atherosclerotic plaques, suggesting a molecular link between NADPH oxidase and PRX-1/TRX levels in atherosclerosis.

To further delineate the potential association of NADPH oxidase and PRX-1/TRX levels, in vitro studies were performed with monocytes/macrophages due to the association between these molecules that we observed both, in circulating PBMC and plaque macrophages. We have shown that when human monocytes were stimulated with PMA, a known NADPH oxidase activity inducer (16), PRX-1/TRX intracellular levels decreased, whereas PRX-1/TRX extracellular levels increased. In our study, apocynin was able to reverse this effect, thus suggesting that PRX-1/TRX levels depends on NADPH oxidase activity/ROS generation. Accordingly, N-acetylcysteine and diphenyliodonium are able to reduce H_20_2 or homocystein-induced TRX expression in human vascular cells (9,27). To ensure the specific involvement of NADPH oxidase and not a general antioxidant effect of apocynin, further studies were performed by silencing Nox2. Knocking-down of Nox2 reversed the decrease of intracellular PRX-1/TRX levels induced by PMA. Collectively, these data support that

oxidative stress induces the release of PRX-1/TRX and suggest the potential involvement of NADPH oxidase-dependent superoxide production on the increased PRX-1/TRX levels observed in atherothrombosis. Nevertheless and given that in the human studies we do not provide direct evidence of the potential role of vascular NADPH oxidases, it is important to point out that the increased phagocytic NADPH oxidase-dependent superoxide production might not necessarily be the only factor responsible for the increased PRX-1/TRX levels. We should also take into account the fact that other enzymes have been proposed as sources of ROS in atherosclerosis such as lipoxygenase, xanthine oxidase, and NO synthase (28).

It has been shown that PRX-1 controls vascular smooth muscle cell proliferation (29). Furthermore, PRX-1 suppresses atherosclerotic lesion formation in apoE–/– mice, probably due to its antiinflammatory activity (30). Similarly, TRX may act as an anti-inflammatory protein. Extracellular TRX has been reported to reduce interleukin 1 beta expression by monocyte-macrophages in inflammatory conditions (31). Circulatory TRX acts as a chemoattractant for monocytes, neutrophils and lymphocytes (32), and inhibits neutrophil migration into inflammatory sites both in vitro and in vivo (33). Accordingly, TRX could be a potential therapeutic target in cardiovascular disorders. In this regard, administration of recombinant TRX prevented myosin-induced myocarditis (34) and smoke-induced emphysema (35) in animal models. Thus, it is tempting to speculate that high PRX-1/TRX extracellular levels observed in atherothrombotic patients could be a defence mechanism to inhibit leukocyte recruitment to the plaque. However, further studies in animal models are required to support this hypothesis.

On the whole, we have shown that circulating PRX-1/TRX is elevated in patients with carotid atherosclerosis and correlates with carotid IMT and NADPH

oxidase-dependent superoxide production in asymptomatic subjects, supporting the potential usefulness of these molecules as biomarkers of oxidative stress in atherosclerosis.

Acknowledgements

We gratefully acknowledge technical assistance by Idoya Rodríguez, Raquel Ros and Ana Montoya. We want to thank Dra. Melina Vega for providing the plasma of carotid atherosclerotic patients and control subjects.

SOURCES OF SUPPORT: This work was supported by the Spanish Ministerio de Ciencia y Tecnología (SAF 2010-21852, SAF2007-62553, SAF2010-20367), CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035; RD06/0014/0008 and EUS2008-03565), European Network (HEALTH F2-2008-200647), European Union (InGeniousHyperCare, grant LSHM-CT-2006-037093) and Sociedad Española de Aterosclerosis. This project was also funded through the agreement between the Fundation for Applied Medical Research (FIMA) and "UTE project CIMA".

No disclosure

REFERENCES

1. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. Am J Cardiol. 2003;91:7A–11A.

2. Lu Y, Wahl LM. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. J Immunol. 2005;175:5423-9.

3. World CJ, Yamawaki H, Berk BC. Thioredoxin in the cardiovascular system. J Mol Med. 2006;84:997-1003.

4. Leavey PJ, Gonzalez-Aller C, Thurman G, Kleinberg M, Rinckel L, Ambruso DW, Freeman S, Kuypers FA, Ambruso DR. A 29-kDa protein associated with p67phox expresses both peroxiredoxin and phospholipase A2 activity and enhances superoxide anion production by a cell-free system of NADPH oxidase activity. J Biol Chem. 2002;277:45181-7.

5. Powis G, Montfort WR. Properties and biological activities of thioredoxins. Annu Rev Biophys Biomol Struct. 2001;30:421-55.

6. Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science. 2003; 300: 650-653.

7. Rubartelli A, Bajetto A, Allavena G, Wollman E, Sitia R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. J Biol Chem. 1992;267:24161-4.

8. Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radic Biol Med. 2005;38:1543-52

9. Kondo N, Ishii Y, Kwon YW, Tanito M, Horita H, Nishinaka Y, Nakamura H, Yodoi J. Redox-sensing release of human thioredoxin from T lymphocytes with negative feedback loops. J Immunol. 2004;172:442-8.

10. Miyamoto S, Sakamoto T, Soejima H, Shimomura H, Kajiwara I, Kojima S, Hokamaki J, Sugiyama S, Yoshimura M, Ozaki Y, Nakamura H, Yodoi J, Ogawa H. Plasma thioredoxin levels and platelet aggregability in patients with acute myocardial infarction. Am Heart J. 2003;146:465-71.

11. Hokamaki J, Kawano H, Soejima H, Miyamoto S, Kajiwara I, Kojima S, Sakamoto T, Sugiyama S, Yoshimura M, Nakamura H, Yodoi J, Ogawa H. Plasma thioredoxin levels in patients with unstable angina. Int J Cardiol. 2005;99:225-31.

12. Martinez-Pinna R, Ramos-Mozo P, Madrigal-Matute J, Blanco-Colio LM, Lopez JA, Calvo E, Camafeita E, Lindholt JS, Meilhac O, Delbosc S, Michel JB, Vega de Ceniga M, Egido J, Martin-Ventura JL. Identification of Peroxiredoxin-1 as a Novel Biomarker of Abdominal Aortic Aneurysm. Arterioscler Thromb Vasc Biol. 2011 Jan 27.

13. Dworakowski R, Anilkumar N, Zhang M, Shah AM. Redox signalling involving NADPH oxidase-derived reactive oxygen species. Biochem Soc Trans. 2006;34(Pt 5):960-4.

14. Lassègue B, Griendling KK. NADPH oxidases: functions and pathologies in the vasculature. Arterioscler Thromb Vasc Biol. 2010;30:653-61.

15. Zalba G, Beloqui O, San José G, Moreno MU, Fortuño A, Díez J. NADPH oxidase-dependent superoxide production is associated with carotid intima-media thickness in subjects free of clinical atherosclerotic disease. Arterioscler Thromb Vasc Biol. 2005;25:1452-7.

16. Zalba G, Fortuño A, Orbe J, San José G, Moreno MU, Belzunce M, Rodríguez JA, Beloqui O, Páramo JA, Díez J. Phagocytic NADPH oxidase-dependent superoxide production stimulates matrix metalloproteinase-9: implications for human atherosclerosis. Arterioscler Thromb Vasc Biol. 2007;27:587-93

17. Fortuño A, Bidegain J, San José G, Robador PA, Landecho MF, Beloqui O, Díez J, Zalba G. Insulin resistance determines phagocytic NADPH oxidase overactivation in metabolic syndrome patients. J Hypertens. 2009;27:1420-30

18. San José G, Bidegain J, Robador PA, Díez J, Fortuño A, Zalba G. Insulin-induced NADPH oxidase activation promotes proliferation and matrix metalloproteinase activation in monocytes/macrophages. Free Radic Biol Med. 2009;46:1058-67.

19. Martín-Ventura JL, Ortego M, Esbrit P, Hernández-Presa MA, Ortega L, Egido J. Possible role of parathyroid hormone-related protein as a proinflammatory cytokine in atherosclerosis. Stroke. 2003;34:1783-9.

20. Mayr M, Chung YL, Mayr U, Yin X, Ly L, Troy H, Fredericks S, Hu Y, Griffiths JR, Xu Q. Proteomic and metabolomic analyses of atherosclerotic vessels from apolipoprotein E-deficient mice reveal alterations in inflammation, oxidative stress, and energy metabolism. Arterioscler Thromb Vasc Biol. 2005; 25: 2135–2142

21. Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. J Biol Chem. 2008; 83: 1622-1627.

22. Martinez-Pinna R, Lindholt JS, Blanco-Colio LM, Dejouvencel T, Madrigal-Matute J, Ramos-Mozo P, Vega de Ceniga M, Michel JB, Egido J, Meilhac O, Martin-Ventura JL. Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAAs). A potential link of oxidative stress with AAA evolution. Atherosclerosis. 2010; 212:333-8.

23. Nichols WW, Pepine CJ, O'Rourke MF. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke. N Engl J Med. 1999;340:1762–3.

24. Jikimoto T, Nishikubo Y, Koshiba M, Kanagawa S, Morinobu S, Morinobu A, Saura R, Mizuno K, Kondo S, Toyokuni S, Nakamura H, Yodoi J, Kumagai S. Thioredoxin as a biomarker for oxidative stress in patients with rheumatoid arthritis. Mol Immunol. 2002;38:765-72.

25. Nishihira K, Yamashita A, Imamura T,Hatakeyama K, Sato Y, Nakamura H, Yodoi J, Ogawa H, Kitamura K, Asada Y. Thioredoxin in coronary culprit lesions: possible relationship to oxidative stress and intraplaque hemorrhage. Atherosclerosis. 2008;201:360-7.

26. Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M. Expression of NADH/NADPH oxidase p22phox in human coronary arteries. Circulation 1999; 100:1494-8.

27. Dai J, Wang X, Feng J, Kong W, Xu Q, Shen X, Wang X. Regulatory role of thioredoxin in homocysteine-induced monocyte chemoattractant protein-1 secretion in monocytes/macrophages. FEBS Lett. 2008;582:3893-8.

28. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol. 2005;25:29-38.

29. Jones FS, Meech R, Edelman DB, Oakey RJ, Jones PL. Prx1 controls vascular smooth muscle cell proliferation and tenascin-C expression and is upregulated with Prx2 in pulmonary vascular disease. Circ Res. 2001;89:131-8.

30. Kisucka J, Chauhan AK, Patten IS, Yesilaltay A, Neumann C, Van Etten RA, Krieger M, Wagner DD. Peroxiredoxin1 prevents excessive endothelial activation and early atherosclerosis. Circ Res. 2008;103:598-605.

31. Billiet L, Furman C, Larigauderie G, Copin C, Brand K, Fruchart JC, Rouis M. Extracellular human thioredoxin-1 inhibits lipopolysaccharide-induced interleukin-1beta expression in human monocyte-derived macrophages. J Biol Chem. 2005;280:40310-8.

32. Bertini R, Howard OM, Dong HF, Oppenheim JJ, Bizzarri C, Sergi R, Caselli G, Pagliei S, Romines B, Wilshire JA, Mengozzi M, Nakamura H, Yodoi J, Pekkari K, Gurunath R, Holmgren A, Herzenberg LA, Herzenberg LA, Ghezzi P. Thioredoxin, a redox enzymereleased in infectionandinflammation, is a uniquechemoattractant for neutrophils, monocytes, and T cells. J Exp Med. 1999;189:1783-9.

33. Nakamura H, Herzenberg LA, Bai J, Araya S, Kondo N, Nishinaka Y, Herzenberg LA, Yodoi J. Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. Proc Natl Acad Sci U S A. 2001;98:15143-8.

34. Liu W, Nakamura H, Shioji K, Tanito M, Oka S, Ahsan MK, Son A, Ishii Y, Kishimoto C, Yodoi J. Thioredoxin-1 ameliorates myosin-induced autoimmune myocarditis by suppressing chemokine expressions and leukocyte chemotaxis in mice. Circulation. 2004;110:1276-83.

35. Sato A, Hoshino Y, Hara T, Muro S, Nakamura H, Mishima M, Yodoi J. Thioredoxin-1 ameliorates cigarette smoke-induced lung inflammation and emphysema in mice. J Pharmacol Exp Ther. 2008;325:380-8.

FIGURE LEGENDS

Figure 1. PRX-1 and TRX plasma levels in patients with carotid atherosclerosis.

Increased (A) PRX-1 and (B) TRX plasma levels in patients with carotid atherosclerosis (n=30) compared to healthy controls (n=30)($p<0.05$). Boxes represent 25th and 75th percentiles; line within boxes, median. Error bars mark 10th and 90th percentiles.

Figure 2. PRX-1 and TRX plasma levels in asymptomatic subjects.

Positive correlation of (A) PRX-1 and (B) TRX plasma levels with phagocytic NADPH oxidase-dependent superoxide production. RLU/s, relative light units/second.

Figure 3. Colocalization studies in human carotid atherosclerotic plaques.

Double immunostaining/immunofluorescence was performed for (A) PRX-1(green) and CD68 (brown), (B) PRX-1 (green) and TRX (brown), (C) p22phox (green) and PRX (brown) and (D) p22phox (green) and TRX (brown). Merge representative images are shown (x40).

Figure 4. PRX-1 and TRX expression in human monocytes stimulated with PMA.

THP-1 cells were stimulated with PMA for 24h after preincubation with apocynin (0.5 or 3 mM, $A_{0.5}$ and A_3, respectively). Intracellular and extracellular proteins were analyzed by Western blot with (A) anti-PRX-1 and (B) anti-TRX antibodies. Tubulin was used as loading control.

Figure 5. PRX-1 and TRX expression in Nox2 siRNA transfected macrophages.

(A) Raw264.7 cells were transfected with nonspecific scramble or Nox2 siRNA. Specific reduction of Nox2 protein is shown by Western blot. (B) Raw264.7 cells transfected with scramble or Nox2 siRNA were treated with PMA and NADPH oxidase activity was determined. (C) Raw264.7 cells were stimulated with PMA for 24h and intracellular proteins were analyzed by Western blot with anti-PRX-1 anti-

TRX antibodies. Tubulin was used as loading control. Data represent means±S.E.M. of 4 independent experiments, each performed in duplicate. * $p<0.05$ compared with the control (cells without PMA) within the same interference experiment.

Table 1. Correlation Coefficients of Mean Carotid IMT, PRX levels and TRX levels with Clinical and Laboratory Parameters in the Asymptomatic Population

	PRX-1		TRX		carotid IMT	
	r	*P*	*r*	*P*	*r*	*P*
Age, y	---	---	---	---	---	---
BMI, kg/m²	0.186	0.097	0.169	0.132	0.179	0.081
SBP, mm Hg	0.063	0.576	0.145	0.200	0.284	0.005
DBP, mm Hg	0.156	0.168	0.195	0.083	0.129	0.214
Glucose, mg/dL	0.052	0.646	0.043	0.706	0.087	0.400
Total cholesterol, mg/dL	0.178	0.112	0.153	0.174	0.035	0.733
HDL cholesterol, mg/dL	-0.033	0.768	0.009	0.936	-0.117	0.255
LDL cholesterol, mg/dL	0.150	0.200	0.108	0.355	0.116	0.278
Triglycerides, mg/dL	0.175	0.118	0.134	0.234	0.092	0.373
Plasma PRX-1, ng/mL	-	-	0.889	<0.001	0.306	0.005
Plasma TRX, ng/ml	0.889	<0.001	-	-	0.363	0.001
Carotid IMT, mm	0.306	0.005	0.363	0.001	-	-

Correlations and *P* values from Pearson correlation coefficient. BMI=body mass index, SBP= systolic blood pressure, DBP=diastolic blood pressure, HDL= high density lipoprotein, LDL=low density lipoprotein, PRX=peroxiredoxin, TRX=thioredoxin, IMT=intima media thickness.

24

Figure 1

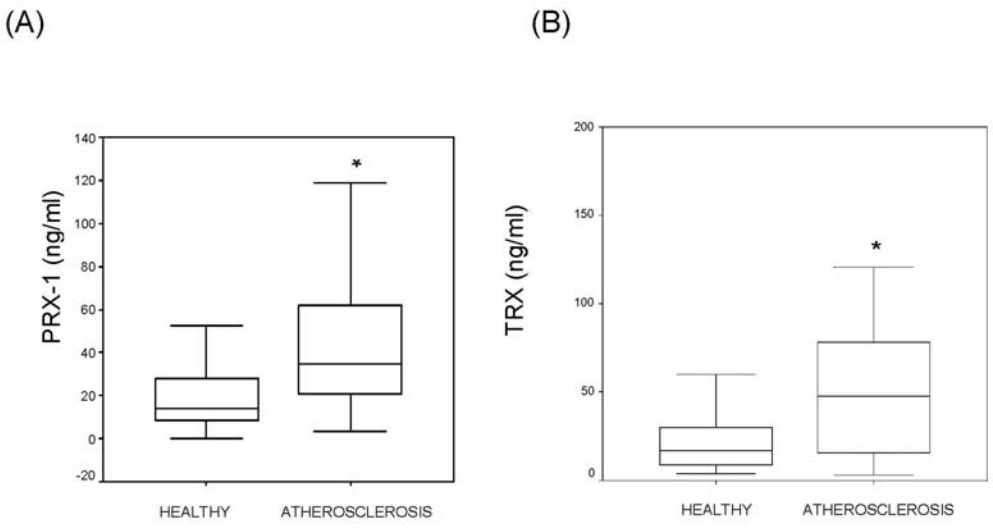


Figure 2

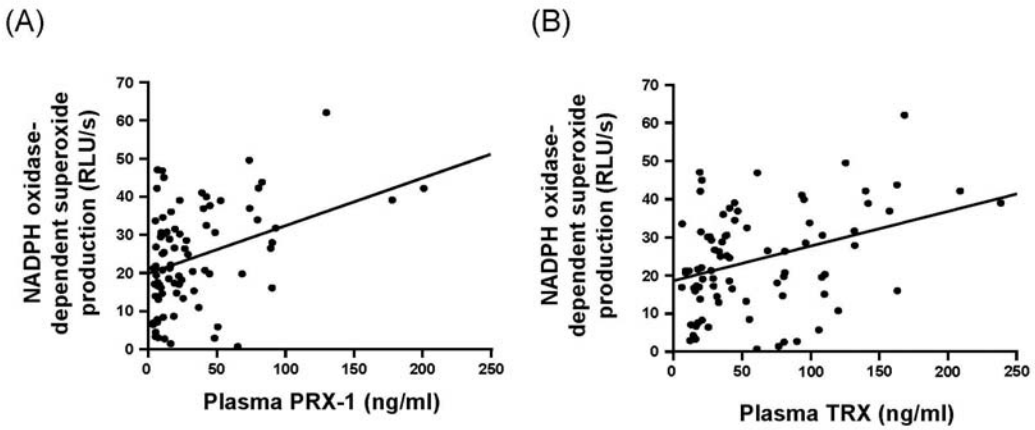


Figure 3

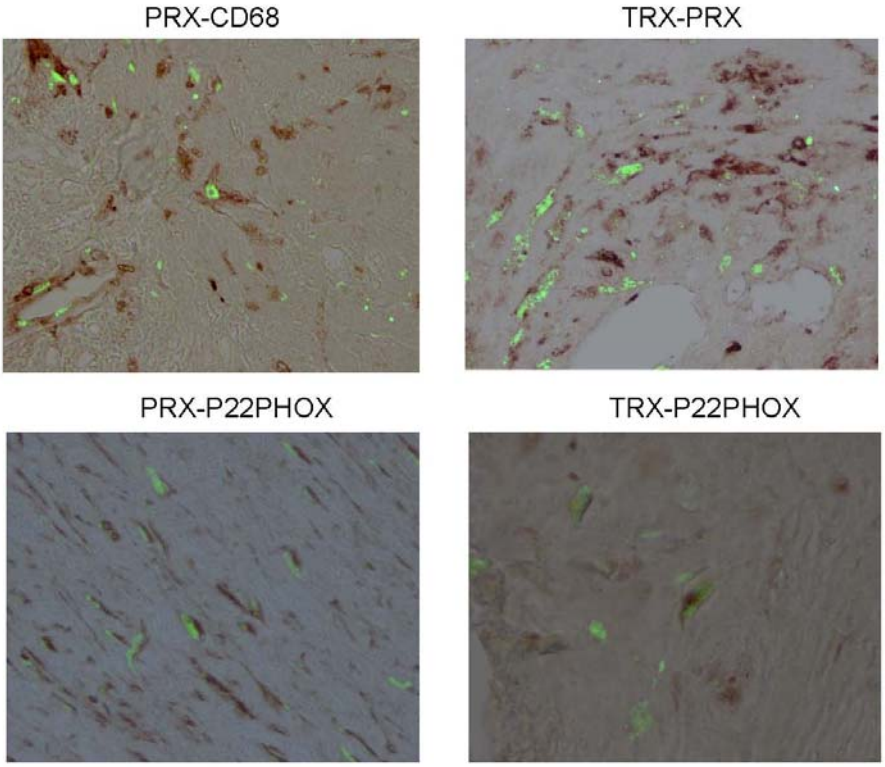


Figure 4

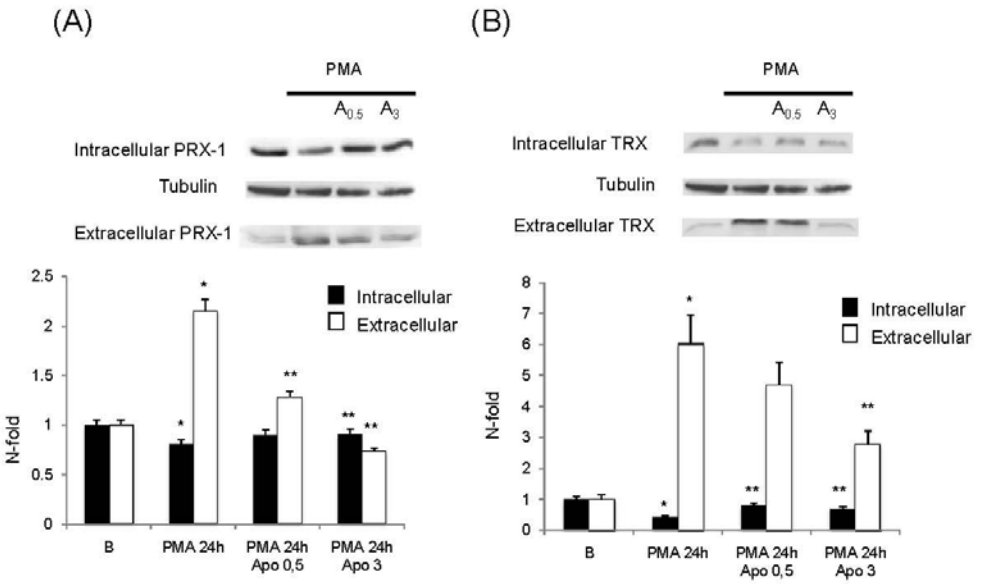
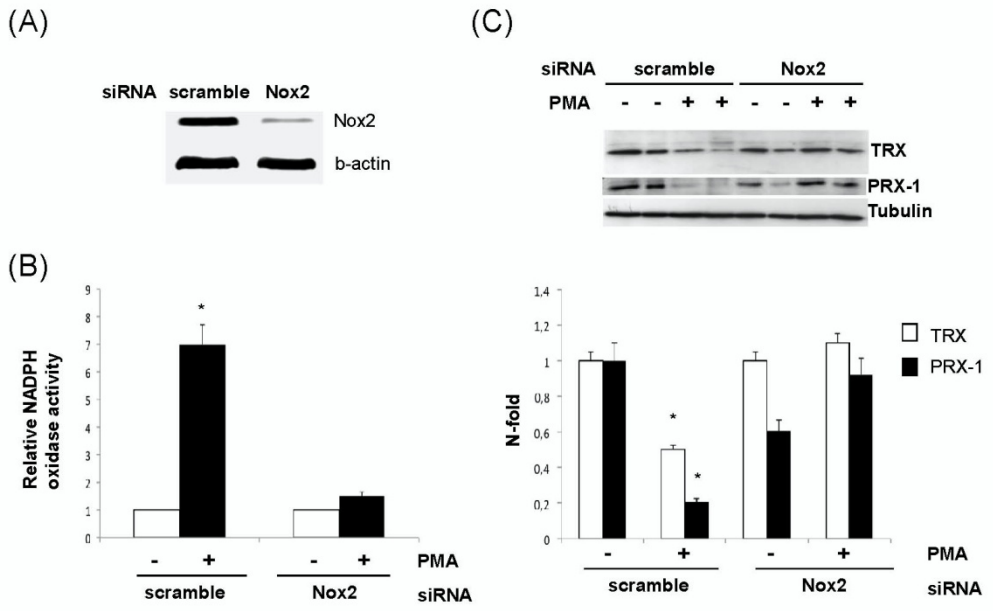
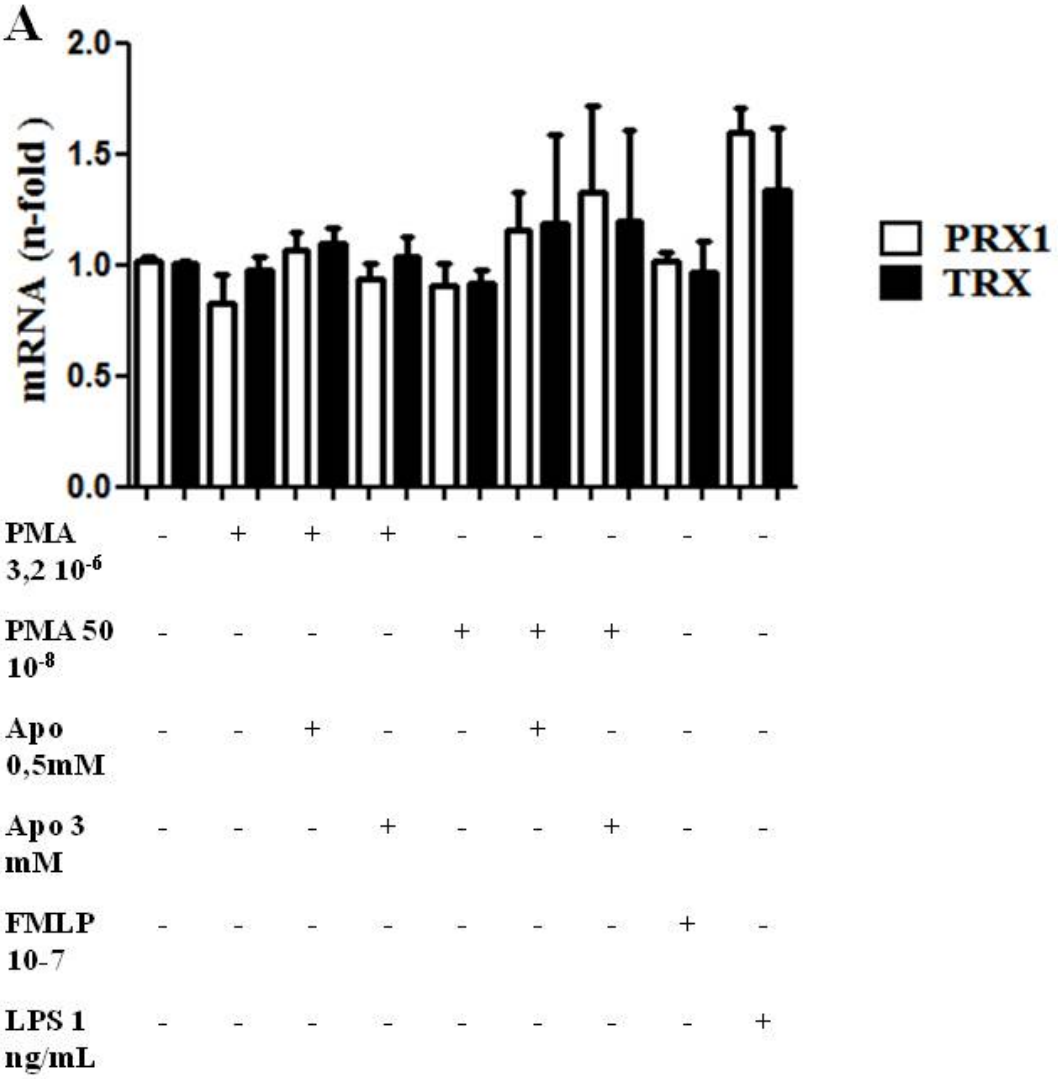


Figure 5

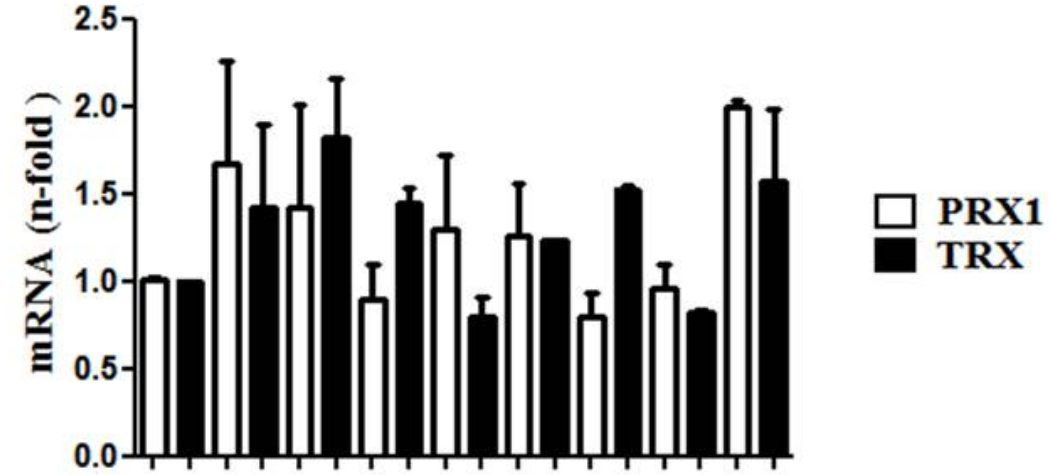


SUPPLEMENTAL FIGURE 1

Supplemental Figure 1 (online).- PRDX1/TRX mRNA levels under different stimuli. (A) PRDX1/TRX mRNA quantification by real-time PCR in THP1 stimulated with different compounds at 3h (B) and at 24h. Values shown are means ± SEM of three independent experiments.



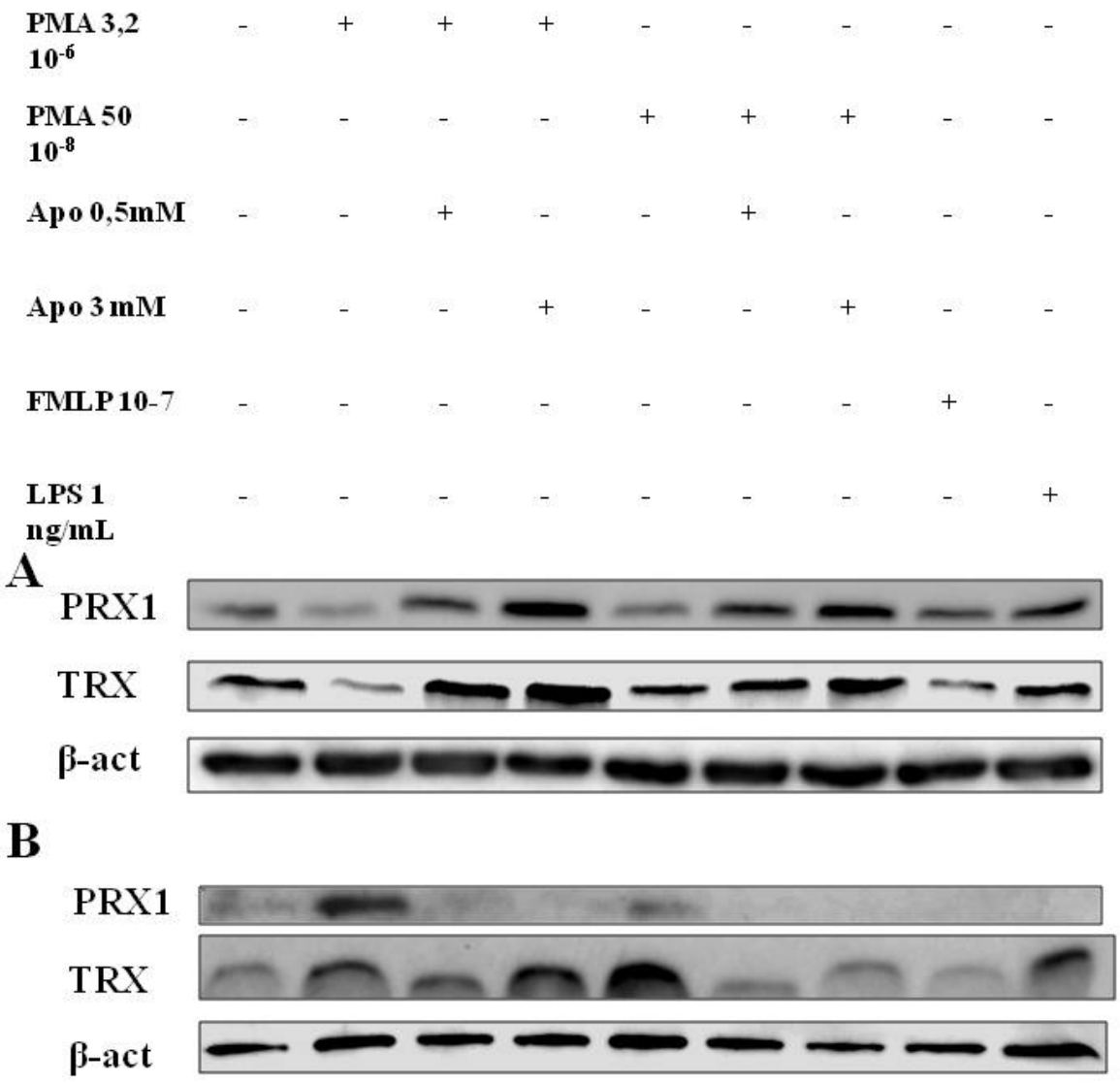
B



PMA 3,2 10^{-6}	-	+	+	+	-	-	-	-	-
PMA 50 10^{-8}	-	-	-	-	+	+	+	-	-
Apo 0,5mM	-	-	+	-	-	+	-	-	-
Apo 3 mM	-	-	-	+	-	-	+	-	-
FMLP 10-7	-	-	-	-	-	-	-	+	-
LPS 1 ng/mL	-	-	-	-	-	-	-	-	+

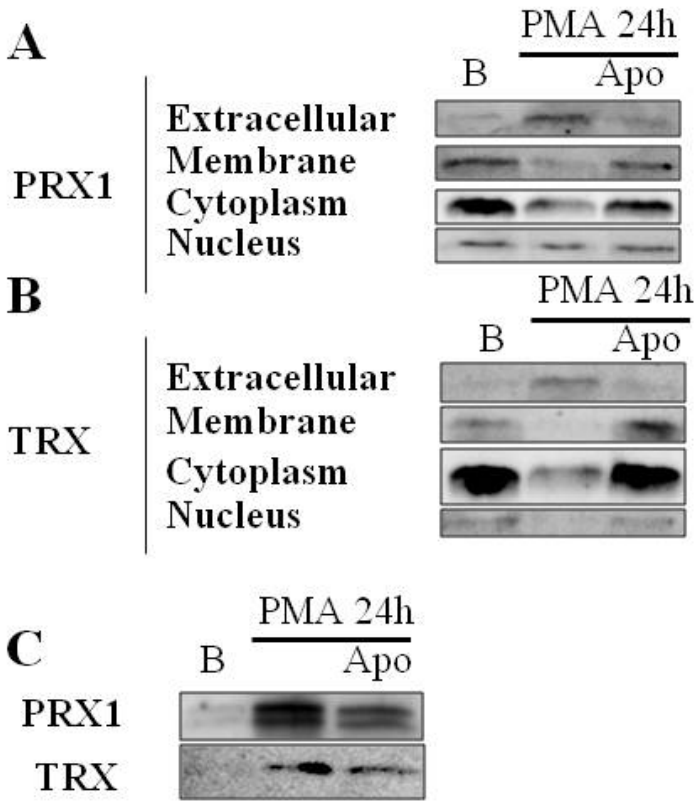
SUPPLEMENTAL FIGURE 2

Supplemental Figure 2 (online).- PRX1/TRX protein levels under different stimuli. (A) Representative immunoblots of intracellular PRX1, TRX and β-act protein in THP1 stimulated with different compounds at 24h (B) and of extracellular PRX1, TRX and β-act protein.



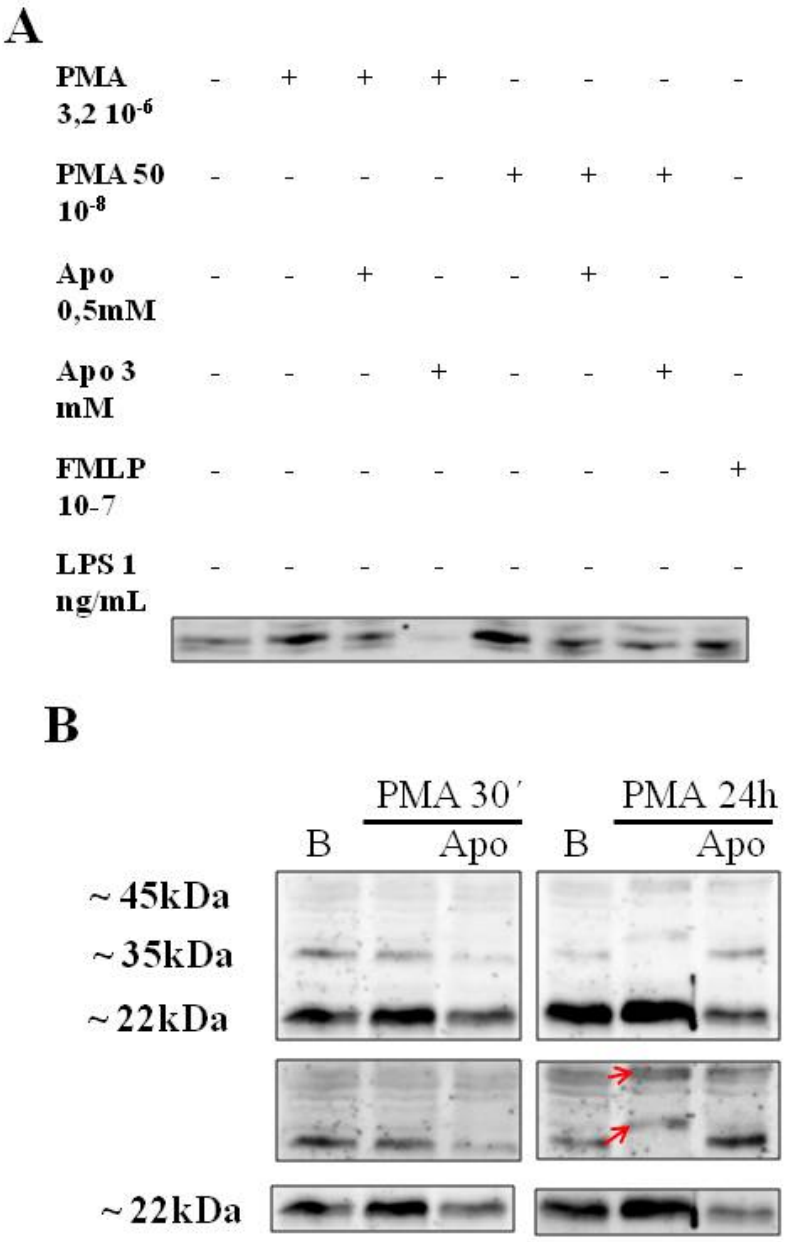
SUPPLEMENTAL FIGURE 3

Supplemental Figure 3 (online).- Cellular location of PRX1/TRX. (A) Representative immunoblots of PRX1 protein levels in THP1 cells stimulated with PMA (3,2 uM, at the times indicated) and under pretreatment with apocynin (0,5mM, 30´). (B) Representative immunoblots of TRX protein levels in THP1 cells stimulated with PMA (3,2 uM, at the times indicated) and under pretreatment with apocynin (0,5mM, 30´). (C) Representative immunoblots of PRX1/TRX protein levels in exosomes derived from THP1 cells stimulated with PMA (3,2 uM, 24h) and under pretreatment with apocynin (0,5mM, 30´). (E) Representative immunoblots of PRX1/TRX protein levels in free-exosomes supernatants derived from THP1 cells stimulated with PMA (3,2 uM, 24h) and under pretreatment with apocynin (0,5mM, 30´).



SUPPLEMENTAL FIGURE 4

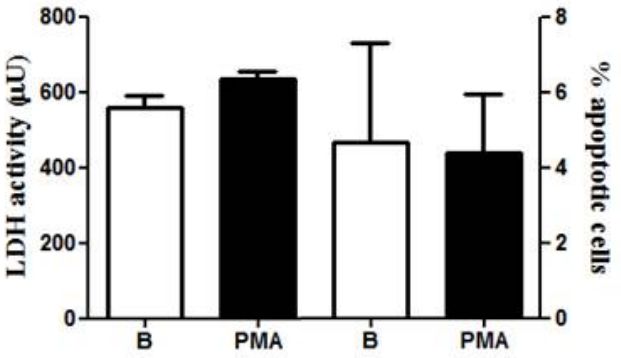
Supplemental Figure 3 (online).- Oxidative status of PRX1/TRX (A) Representative immunoblot of oxydized total intracellular PRX1 in THP1 stimulated with different compounds at 24h. (B) Representative immunoblots of oxydized PRX1 cytoplasmic protein levels in THP1 cells stimulated with PMA (3,2 uM, at the times indicated) and under pretreatment with apocynin (0,5mM, 30´).



SUPPLEMENTAL FIGURE 5

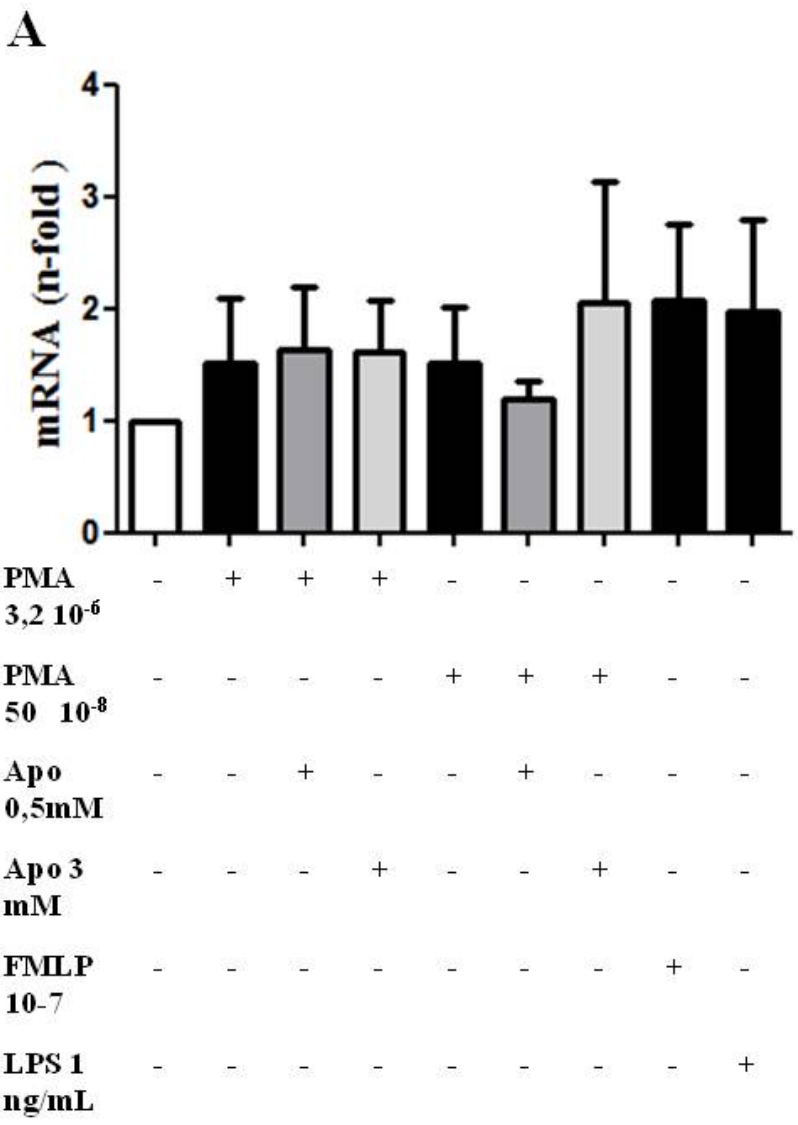
Supplemental Figure 5 (online).- Effect of PMA on THP1 cell death, either on necrosis or apoptosis. Values shown are means ± SEM of three independent experiments.

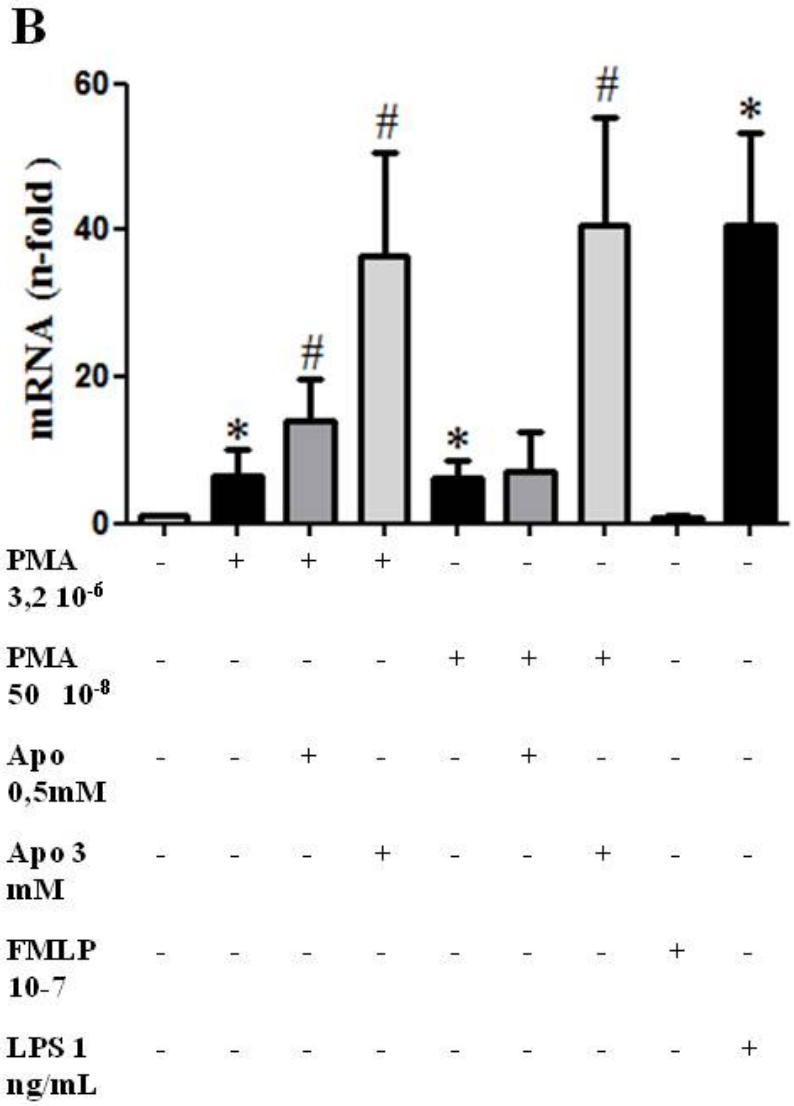
A

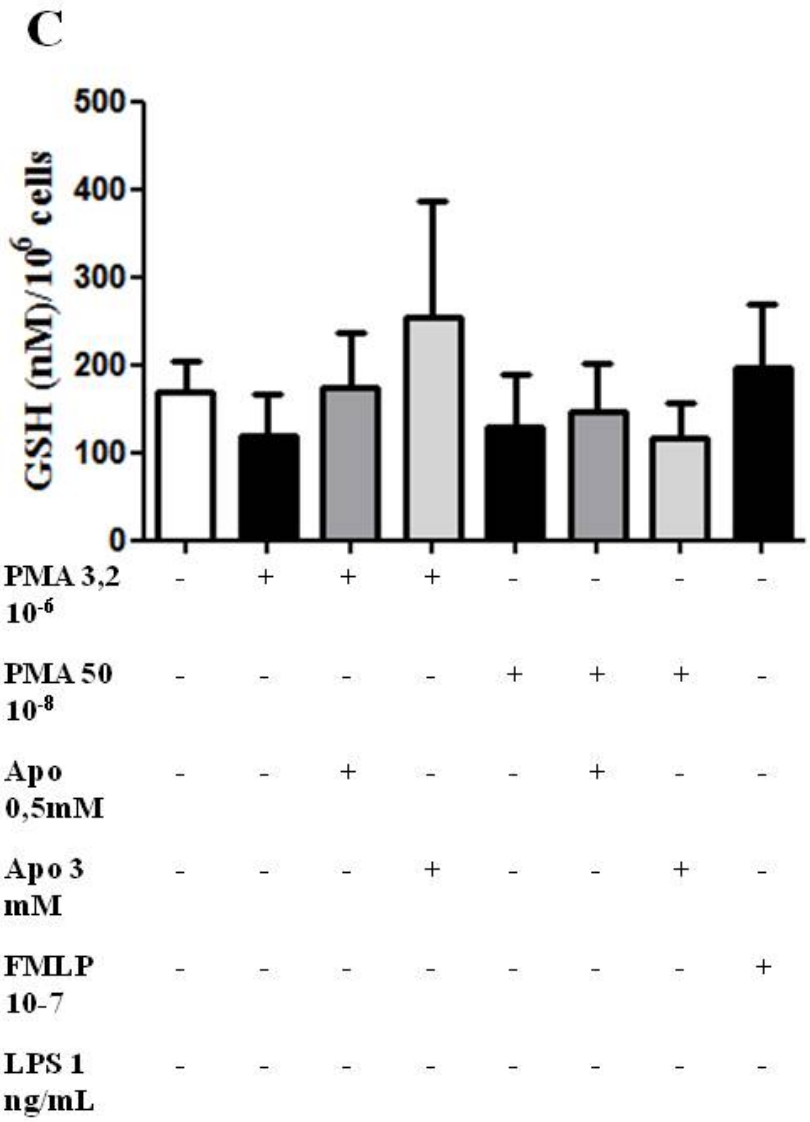


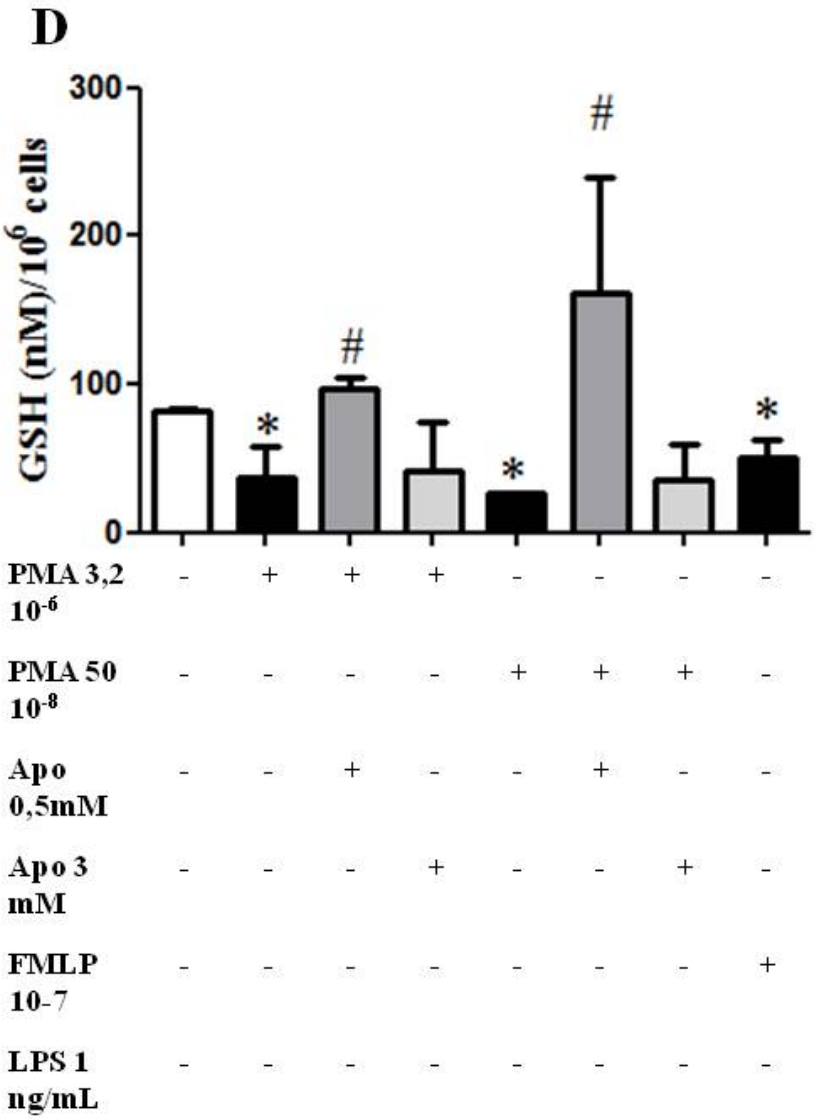
SUPPLEMENTAL FIGURE 6

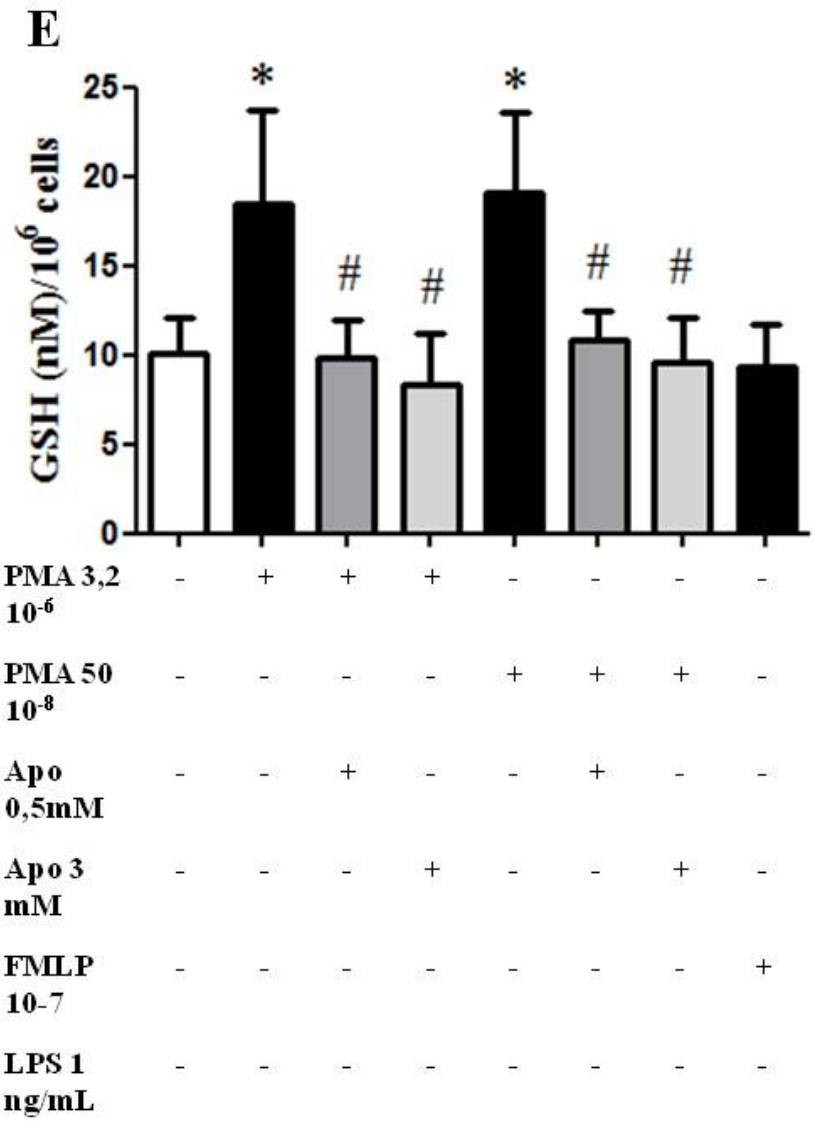
Supplemental Figure 6 (online).- Antioxidant response in THP1 cells un different stimuli. (A) MnSOD mRNA quantification by real-time PCR in THP1 stimulated with different compounds at 3h (B) and at 24h. (C) Intracellular ratio of GSH/GSSG in THP1 stimulated with different compounds at 30´ (D) and at 24h. (E) Extracellular ratio of GSH/GSSG in THP1 stimulated with different compounds at 24h. Values shown are means ± SEM of three independent experiments. * p <0,05 vs Basal, # p < 0,05 vs PMA.




B
mRNA (n-fold)
60
40
20
0
*
#
#
*
#
*
PMA 3,2 10-6 - + + + - - - - -
PMA 50 10-8 - - - - + + + - -
Apo 0,5mM - - + - - + - - -
Apo 3 mM - - - + - - + - -
FMLP 10-7 - - - - - - - + -
LPS 1 ng/mL - - - - - - - - +


C
GSH (nM)/10^6 cells
500
400
300
200
100
0
PMA 3,2 10-6 - + + + - - - -
PMA 50 10-8 - - - - + + + -
Apo 0,5mM - - + - - + - -
Apo 3 mM - - - + - - + -
FMLP 10-7 - - - - - - - +
LPS 1 ng/mL - - - - - - - -


D
GSH (nM)/10^6 cells
300
200
100
0
#
#
*
*
*
PMA 3,2 10-6 - + + + - - - -
PMA 50 10-8 - - - - + + + -
Apo 0,5mM - - + - - + - -
Apo 3 mM - - - + - - + -
FMLP 10-7 - - - - - - - +
LPS 1 ng/mL - - - - - - - -


E
GSH (nM)/10^6 cells
25
20
15
10
5
0
*
*
#
#
#
#
PMA 3,2 10-6 - + + + - - - -
PMA 50 10-8 - - - - + + + -
Apo 0,5mM - - + - - + - -
Apo 3 mM - - - + - - + -
FMLP 10-7 - - - - - - - +
LPS 1 ng/mL - - - - - - - -

IV. DISCUSIÓN

La prevalencia de la aterosclerosis aumenta rápidamente en la mayor parte de los países, especialmente, especialmente en aquellos que han modificado sus hábitos hacia aquellos propios de la vida occidental [135]. Por ello, es necesario desarrollar terapias que puedan modular o frenar el desarrollo de esta enfermedad. En esta tesis hemos demostrado que el uso de fármacos que puedan aumentar los niveles de CSPs ateroprotectoras, así como la inhibición de ciertas rutas de señalización implicadas en la enfermedad aterosclerótica, podrían ser de utilidad clínica. Asimismo, hemos analizado el posible papel como potenciales biomarcadores diagnosticos de las CSPs tanto a nivel intracelular como a nivel circulatorio en aterotrombosis.

4.1. . Efectos ateroprotectores de los inhibidores de HSP90 a través de la disminución de la respuesta inmuno-inflamatoria

En el apartado 3.1 de Materiales, Métodos y Resultados analizamos en primer lugar los niveles de HSP70 y HSP90 en arterias carótidas de pacientes con aterosclerosis avanzada. Estudios previos realizados en arterias coronarias de pacientes que sufrieron infarto de miocardio mostraron que la rotura del ateroma sucede generalmente en la zona de los hombros [105], un área caracterizada por un alto contenido de células inflamatorias, activación de NF-κB y expresión de MCP-1 [142, 144]. Asimismo, se ha descrito que la rotura de la placa aterosclerótica sucede frecuentemente en aquellas zonas donde la placa fibrosa es más delgada y en aquellas áreas en las que el componente inflamatorio es mayor. Los resultados muestran unos altos niveles de expresión proteica de HSP90 en placas avanzadas de pacientes con aterosclerosis, especialmente en la zona de los hombros de la placa. Además, los niveles de HSP90 fueron mayores en aquellas placas cuya capa fibrosa era más delgada, lo que sugiere que HSP90 podría jugar un papel importante en la inestabilidad de la placa avanzada. Durante la preparación de este manuscrito y en línea con los datos que nosotros habíamos hallado, Businaro *et al* publicaron un artículo que describía la sobreexpresión de HSP90 tanto en placas como en suero de pacientes con aterosclerosis [29]. Estos autores sugieren que la elevada expresión de HSP90 en placas ateroscleróticas podría ser el responsable de iniciar una respuesta específica autoinmune que exacerbaría la cascada inflamatoria mediante la estimulación de la inmunidad innata y adquirida. Además, los linfocitos T específicos para HSP90 mostraban un perfil pro-inflamatorio tipo Th1. Debido a que los linfocitos Th1 producen citoquinas como IFN-γ, IL-2 y TNF-β que son responsables de la activación de macrófagos y de la inmunidad celular [73], Businaro *et al* sugieren un papel para HSP90 en el mantenimiento de los mecanismos inflamatorios que subyacen a la aterogénesis. Nuestros estudios amplían estos datos y ponen de manifiesto que HSP90 puede ser

una posible diana terapéutica en aterosclerosis. Hasta el momento, los inhibidores de HSP90 se han estudiado principalmente en cáncer [229], aunque se ha demostrado en diferentes tipos celulares que estas drogas son capaces de bloquear la actividad de ciertos mediadores pro-inflamatorios [140, 254], y también existen evidencias sobre su posible papel beneficioso en varios modelos experimentales de enfermedades inflamatorias como artritis reumatoide [204], uveítis inducida por endotoxinas [184] y sepsis [35]. Además, la inhibición de HSP90 mediante el 17-AAG modula otros procesos implicados en aterosclerosis como la migración endotelial y angiogénesis, debido a su interacción con Akt y eNOS [236]. Dado que la inflamación excesiva desequilibra la balanza hacia la progresión de la aterosclerosis, las estrategias que puedan prevenir esta respuesta patológica podrían ser potencialmente beneficiosas. Hemos observado que los inhibidores de HSP90 disminuyen la expresión (MCP-1 e IL-6) y activación (STAT3 y NF-κB) de mediadores inflamatorios tanto *in vitro* como *in vivo* (Figura 8). Así, los efectos beneficiosos de HSP90 en las enfermedades inflamatorias podrían ser debido a su doble actividad: por un lado la degradación de proteínas cliente implicadas en diferentes rutas de señalización pro-inflamatorias y, por otro la sobreexpresión de proteínas HSPs anti-inflamatorias, como HSP70. Dentro de las proteínas cliente de HSP90 implicadas en enfermedades inflamatorias, STAT y NF-κB son los ejemplos más representativos. JAK/STAT es una ruta de señalización importante que se encuentra regulada por receptores de citoquinas y modula la iniciación/progresión de la aterosclerosis y el remodelado en respuesta al daño [1, 218]. La activación de JAK/STAT se ha descrito previamente en lesiones ateroscleróticas y en células vasculares en cultivo sometidas a condiciones pro-inflamatorias [64, 170, 197]. En ratones deficientes en STAT3 de manera condicional, la formación de estrías grasas se redujo, en comparación con ratones control [64]. Por el contrario, la inhibición de los reguladores negativos de STAT3 mediante oligonucleótidos antisentido inicia la activación de STAT3 y, en consecuencia, agrava el proceso inflamatorio en ratones ApoE$^{-/-}$ [170]. Estos datos apoyan los resultados que hemos obtenido en esta tesis ya que hemos observado que la prevención en la activación de STAT3 mediante los inhibidores de HSP90 es capaz de disminuir la respuesta inmuno-inflamatoria en células vasculares, macrófagos y en placas ateroscleróticas de ratones.

La ruta de señalización de NF-κB tiene un papel crucial en la mediación de las respuestas inmunes e inflamatorias. La activación de NF-κB requiere la fosforilación de IκB por el complejo de cinasas IKK, lo que favorece la ubiquitinación y degradación de IκB por el proteosoma, permitiendo a NF-κB translocarse al núcleo y regular transcripcionalmente la expresión de diferentes genes pro-inflamatorios (por ej. MCP-1). Se ha observado previamente que la inhibición de NF-κB disminuyó los procesos inflamatorios en el modelo de aterosclerosis ApoE^{-}

/- [41, 136]. Otras aproximaciones como oligodeoxinucleótidos antisentido para NF-κB han sido utilizadas de manera exitosa en modelos animales de enfermedades vasculares [273]. Es interesante resaltar que IKK al ser una proteína cliente de HSP90, forma un complejo con esta chaperona, de esta forma el uso de los inhibidores de HSP90 bloquea la función de IKK, y la posterior activación de NF-κB [155]. En concordancia con estos datos, hemos observado que los inhibidores de HSP90 modulan la activación de NF-κB inducida por citoquinas pro-inflamatorias en células vasculares y macrófagos (Figura 8). Así, los inhibidores de HSP90 disminuyeron la activación de NF-κB, los niveles de MCP-1 y el infiltrado de monocitos/macrófagos en placas ateroscleróticas. Estos resultados concuerdan con trabajos previos en los que los inhibidores de HSP90 atenuaban NF-κB en otras enfermedades inflamatorias [35, 184].

Las proteínas de choque térmico están implicadas en la protección de las células frente a diversos tipos de daño. Aunque tradicionalmente el mecanismo de esta protección está asociado a su función chaperona, se sabe que ciertas HSPs, como HSP70, también presentan propiedades anti-inflamatorias en diferentes condiciones patológicas [271]. Así, HSP70 es capaz de inhibir la adhesión y reclutamiento de leucocitos en modelos *in vivo* [87], disminuir el número de macrófagos activados e inhibir la activación de NF-κB en un modelo de inflamación cerebral [281]. En un modelo de colitis, Tanaka *et al*, demostraron que la sobreexpresión de HSP70 en ratones transgénicos inhibía la expresión de numerosas citoquinas, incluyendo la IL-6. Otras aproximaciones, como la inducción de HSP70 mediante el consumo de bajas dosis de alcohol sugieren que dicho efecto cardioprotector estaba mediado por un aumento de HSP70 y HO-1 [213]. Del mismo modo, se ha observado el efecto beneficioso de la inducción de HSP70 con 17-AAG en el tratamiento de la encefalomielitis autoinmune experimental a través de la supresión de las respuestas inflamatorias de la glía [44]. Nuestros resultados sugieren que la inducción de HSP70 mediante el tratamiento con 17-AAG/17-DMAG podría ser un mecanismo adicional para disminuir la inflamación en células vasculares y placas ateroscleróticas (Figura 8). Asimismo, la relevancia clínica de nuestros resultados está relacionada con la evidencia cada vez mayor de que las HSPs podrían ser posibles dianas terapéuticas en el tratamiento de diferentes enfermedades [229]. Para este objetivo, se debería utilizar 17-DMAG ya que es un derivado más potente y con mayor solubilidad que el 17-AAG [78, 227], pudiendo ser administrado oralmente.

Como resumen de esta primera parte, nuestros resultados demuestran que los inhibidores de HSP90 interfieren con el desarrollo de la placa de ateroma, pudiendo promover la estabilidad de la misma a través de la reducción de los factores inflamatorios.

4.2. La inhibición de HSP90 disminuye el estrés oxidativo y la diferenciación monocito-macrófago en aterosclerosis experimental

El estrés oxidativo está implicado en todas las fases del proceso aterogénico, desde etapas iniciales hasta las complicaciones, que desembocan en eventos clínicos debido a la rotura de placas avanzadas. Por lo tanto, las terapias que prevengan el aumento patológico de la producción de ROS en el sistema vascular resultan, sin duda, beneficiosas. Ya que las ROS median la inter-relación positiva entre el estrés oxidativo y la inflamación en la pared arterial, en el apartado 3.2 de esta tesis analizamos en primer lugar la producción de superóxido en un modelo *in vivo* de aterosclerosis de $ApoE^{-/-}$. Los estudios con la sonda fluorescente sensible a la producción de ión superóxido (dihidroetidio, DHE) muestran que las placas ateroscleróticas de los ratones tratados con el 17-DMAG sufrían un menor estrés oxidativo en comparación con los controles. Los experimentos *in vitro* confirmaron el efecto antioxidante de la inhibición de HSP90 (Figura 8). Así, en CMLVs de rata tratadas con TNF-α, y dosis bajas (no citotóxicas) de 17-DMAG redujo parcialmente los niveles de ROS inducidos por el TNF-α.

Como ya se comentó anteriormente, dos de los efectos pleiotrópicos de la inhibición de HSP90 por el 17-DMAG son la inducción en la síntesis de HSPs y la degradación de proteínas cliente de HSP90, modulando de esta forma numerosas rutas de señalización. Así, mostramos cómo el tratamiento de CMLVs con 17-DMAG aumenta la expresión génica y proteica de HSP27 y HSP70 (Figura 8). Un dato interesante que obtuvimos del análisis de las placas ateroscleróticas de ratón de nuestro modelo es que existe una relación inversa entre la producción local de ión superóxido y la expresión de HSP70. Para estudiar los mecanismos que subyacen a esta correlación inversa, realizamos estudios de silenciamiento genético *in vitro* mediante el uso de un siRNA específico para HSP70 en CMLVs. De acuerdo con los datos *in vivo*, la reducción en los niveles de HSP70 en CMLVs produjo un marcado incremento en la producción de ROS (Figura 8). En este contexto, estudios previos habían mostrado que la sobreexpresión de HSP70 tiene efectos antioxidantes en un modelo de aterosclerosis en ratas [168], protegiendo a las células a nivel mitocondrial frente al estrés oxidativo inducido por H_2O_2 [181] y manteniendo los niveles de GSH [268]. Además puede representar un mecanismo de defensa en las células endoteliales y en las células vasculares frente a las LDLs oxidadas [282, 283]. Asimismo, los efectos antioxidantes del tratamiento con resveratrol en aterosclerosis van unidos al aumento en los niveles de HSP27 [253], la modulación en los niveles de GSH [192] y la disminución en la activación de NF-ϰB [25]. Las perturbaciones en la estructura del citoesqueleto son una de las mayores consecuencias del estrés oxidativo intenso [153]. Las HSPs pequeñas, como la HSP27 así como otras HSPs, HSP90 o la

HSP70, protegen a los filamentos intermedios y microfilamentos, previniendo de esta forma daño en el centrosoma [130, 158].

Por otro lado, los efectos duales de la inhibición de HSP90 incluyen la modulación de numerosas rutas de señalización implicadas en el estrés oxidativo, como por ejemplo la cascada de MAPK, y en particular ERK1/2, una cinasa ampliamente descrita por su papel en el estado redox celular. HSP90 media la fosforilación de ERK1/2 promoviendo su translocación nuclear e incrementando de esta forma la proliferación de las CMLVs de rata en respuesta al estrés oxidativo [133]. En nuestras observaciones *in vivo*, las placas ateroscleróticas de los ratones tratados con 17-DMAG mostraban niveles de activación de ERK1/2 menores que los de ratones control. Este efecto fue confirmado por experimentos *in vitro,* en los que el tratamiento de CMLVs con 17-DMAG fue capaz de disminuir tanto los niveles basales de fosforilación de ERK1/2 como los inducidos por TNF-α (Figura 8). Estos resultados están en línea con trabajos anteriores en los que la inhibición de HSP90 mediante 17-AAG [86] o 17-DMAG [69] reducía la fosforilación de ERK en distintos tipos celulares. La relación entre la producción de ROS y la activación de ERK ha sido previamente descrita en CMLVs [222]. Por tanto, la atenuación de ERK mediada por el 17-DMAG podría inhibir la producción de ROS y otras rutas de señalización implicadas en estrés oxidativo y aterosclerosis reguladas por esta cinasa. Hay que resaltar que el 17-DMAG fue capaz de reducir la actividad NADPH oxidasa en CMLVs, tanto sus niveles basales como los inducidos por TNF-α (Figura 8). Aunque los mecanismos moleculares de la modulación de la NADPH oxidasa no están totalmente claros, es posible que el rápido incremento en la actividad NADPH oxidasa inducida por TNF-α, como se ha mostrado previamente para el PMA [36], pudiera ser debido a un cambio en el estado de afinidad en la unión de p22phox a NOXO1 [50]. Por otro lado, la modulación de la actividad NADPH oxidasa por parte del 17-DMAG se podria producir a través de su unión directa a HSP90, ya que NOX1 es una proteína cliente de HSP90. Debido que la interacción entre HSP90 y la subunidad NOX1 de la NADPH oxidasa se produce a través del extremo C-terminal del residuo NOX1 [36], es posible que la unión del 17-DMAG al bolsillo ATP de la proteína HSP90 impida la interacción HSP90/NOX1. En este sentido, hemos observado que la inhibición de HSP90 mediante el uso de un siRNA específico para HSP90α/β inhibió casi totalmente la producción de ROS dependiente de la NADPH oxidasa en CMLVs. Además, el 17-DMAG podría romper la unión de la región rica en prolinas de p22phox al dominio en tándem SH3 de NOXO1, lo que constituiría un mecanismo adicional de inhibición de ROS, similar al demostrado previamente para el celastrol [96]. El celastrol, es un producto extraído de plantas medicinales tradicionales entre cuyos efectos están la inactivación de Cdc37 y p23 (cochaperonas de HSP90), la inhibición del proteasoma y la activación de HSF1 desencadenando la respuesta

por choque térmico [210]. Hemos encontrado en CMLVs que el 17-DMAG es capaz de reducir la expresión de RNAm y proteína de NOX1 y NOXO1, tanto en condiciones basales como tras estimulación por TNF-α (Figura 8). Esta disminución en los niveles de ambas subunidades de la NADPH no está necesariamente relacionada con el descenso en la actividad NADPH oxidasa, sino que es otro efecto de la inhibición de HSP90 [36], pudiendo ser beneficioso a largo plazo en enfermedades como la aterosclerosis. El tratamiento prolongado con 17-DMAG disminuye los niveles de las subunidades de la NADPH oxidasa, pero los efectos a corto plazo incluyen la modulación de la actividad NAPDH oxidasa, como también han demostrado otros autores para diversos análogos de la geldanamicina [36].

Para intentar profundizar en el efecto que podría tener el tratamiento prolongado con 17-DMAG en otras células del sistema vascular, realizamos experimentos en monocitos humanos. De esta forma, demostramos que el PMA induce la producción de ROS dependiente de NADPH oxidasa, la sobreexpresión de NOX1 y NOXO1 y la diferenciación del monocito a macrófago en la línea celular monocítica THP-1. De acuerdo con nuestros resultados, varios estudios demuestran que NOX1 está aumentada en macrófagos activados por LPS [123], estimula la oxidación de LDLs y es clave para la formación de células espumosas, favoreciendo por tanto los procesos oxidativos e inflamatorios [121]. En este sentido, es interesante resaltar que nuestros resultados demuestran que el 17-DMAG inhibe de manera efectiva la producción de ROS dependiente de de la NAPDH oxidasa y la expresión de NOX1 y NOXO1, junto con la de CD36, un marcador de la diferenciación monocito-macrófago [162] (Figura 8). Así, las consecuencias funcionales en un tratamiento prolongado de la inhibición de HSP90 podría incluir la disminución de la producción de ROS implicada en la diferenciación de monocito a macrófago, un proceso clave en la formación de células espumosas y que favorece el desarrollo y progresión de la lesión [176].

Para profundizar en la relevancia de NOX1 y NOXO1 en aterosclerosis, analizamos la expresión de ambas proteínas en placas humanas de aterosclerosis carotídea. Los niveles de RNAm de NOX1 han sido estudiados previamente en placas ateroscleróticas humanas [228], pero hasta el momento no hay ningún estudio que analizase la localización de las proteínas NOX1 y NOXO1 en placas ateroscleróticas humanas. Nuestros resultados demuestran la expresión de ambas subunidades de la NADPH oxidasa asociadas tanto a CMLVs como a macrófagos de la lesión ateromatosa, principalmente en la zona inflamatoria. De manera similar otras subunidades de la NADPH oxidasa, como p22phox, han sido previamente descritas en placas ateroscleróticas humanas [228]. Además encontramos colocalización de NOX1 y NOXO1 en células positivas para la sonda fluorescente sensible a ión superóxido DHE, lo que sugiere que las células que expresan

altos niveles de estas subunidades son productoras de elevados niveles de ROS. Estos datos sugieren que podría existir una retroalimentación positiva en un escenario de estrés oxidativo crónico entre las ROS derivadas de NOX1 y la formación de macrófagos. Aunque, el papel de NOXO1 en enfermedades vasculares no es tan conocido, se ha descrito que los niveles de NOX1 y NOXO1se incrementan por estimulación con citoquinas pro-aterogénicas como el IFN-γ y el TNF-α en diferentes tipos celulares [106]. Asimismo, describimos una fuerte tinción tanto de NOX1 como de NOXO1 en la zona inflamatoria de placas humanas de aterosclerosis avanzada. Por otro lado, un análisis de genoma completo hecho en modelo murino de enfermedad crónica obstructiva de pulmón, mostró que los niveles de NOXO1 estaban altamente incrementados en aquellos pulmones expuestos al humo de tabaco y LPS, mientras que la expresión de HSP70 (Hspa1b) disminuyó drásticamente. Teniendo en cuenta estos datos, las variaciones en los niveles de NOXO1/HSP70 podrían ser un marcador del desequilibrio en la maquinaria antioxidante [149]. Finalmente, cuantificamos mediante western blot en extractos proteicos obtenidos de placas ateroscleróticas y confirmamos la expresión proteica incrementada de NOX1 y NOXO1 en la región complicada de la placa, compuesta principalmente por células inflamatorias y hemorragia intraplaca, comparada con su zona no complicada, compuesta principalmente por CMLVs y depósitos lipídicos, respectivamente. Todos estos datos subrayan la importancia de las ROS derivadas de NOX1 en un escenario crónico inmune-inflamatorio y oxidativo presente en las lesiones ateroscleróticas avanzadas humanas.

En resumen, estos estudios demuestran que el 17-DMAG es capaz de disminuir la respuesta oxidativa en aterosclerosis, apuntando hacia un posible papel terapéutico del 17-DMAG en enfermedades relacionadas con el estrés oxidativo y la inflamación.

Sin embargo, ya que la inhibición de HSP90 afecta a numerosas rutas de señalización a la vez, otros procesos patológicos como la neoangiogénesis y la apoptosis podrían estar afectados por estas drogas. Por lo tanto, se requieren futuros estudios para clarificar el papel protector de estos fármacos en enfermedades humanas. Hasta el momento el uso de estos inhibidores en ensayos clínicos se ha restringido al campo del cáncer [22, 114, 115, 171, 195], pero como hemos demostrado la sobreexpresión de ciertas HSPs mediante el uso de compuestos farmacológicos podría ser un método prometedor para el tratamiento de la aterosclerosis. Las dosis y la toxicidad que se puedan extraer de los diferentes ensayos clínicos en fase I con diferentes derivados de la geldanamicina que se están llevando a cabo en pacientes con cáncer podrían facilitar la selección de dosis no citotóxicas para su posterior estudio en pacientes con enfermedades no neoplásicas, como las enfermedades cardiovasculares.

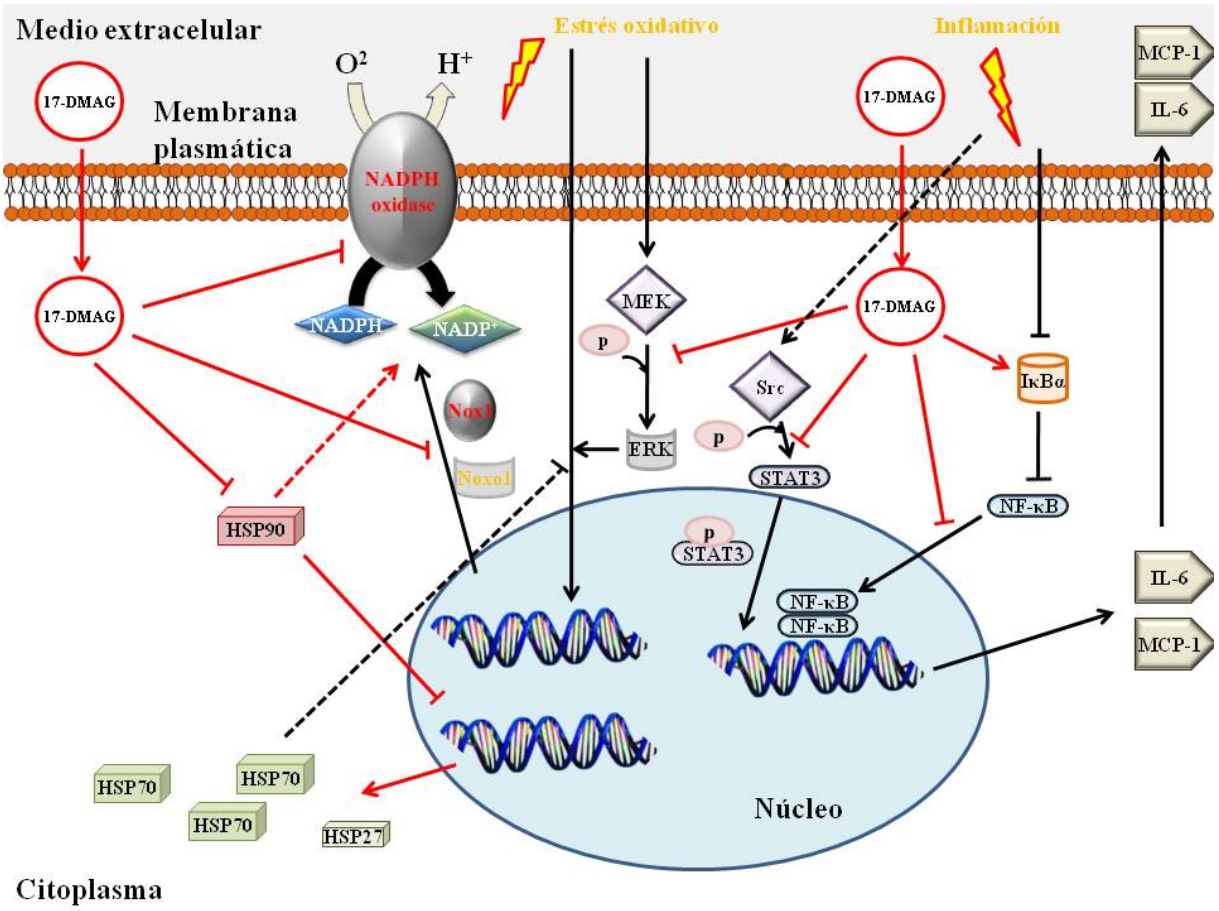


***Figura 8. Esquema de los diferentes efectos estudiados del 17-DMAG en condiciones pro-inflamatorias y de estrés oxidativo* in vitro *e* in vivo.**

4.3. Los niveles circulantes de PRDX-1/TRX aumentan en aterosclerosis; descripción de los mecanismos asociados a su secreción bajo condiciones prooxidantes

Las ROS, como el O_2^-, H_2O_2 y el HO·, consisten en especies formadas por la reducción parcial del oxígeno. Las ROS celulares se generan de manera endógena o pueden producirse a través de la interacción con fuentes exógenas, como compuestos xenobióticos. El estrés oxidativo tiene lugar bien por un aumento en la producción de ROS o bien por un descenso en la capacidad antioxidante celular. El estrés oxidativo provoca daños en ácidos nucleicos, proteínas y/o lípidos, estando implicado en numerosas enfermedades como la aterosclerosis o la diabetes [173]. La NAPDH oxidasa, principal fuente de ROS en el sistema vascular, junto con el sistema de la TRX forman el principal sistema regulador del estado redox celular. Los niveles circulantes de TRX están elevados en pacientes con historia clínica de infarto de miocardio o angina inestable [84, 154]. En esta tesis, a lo largo del apartado 3.3 describimos un incremento tanto en los niveles plasmáticos de PRDX-1 como de TRX en pacientes con aterosclerosis carotídea en comparación con pacientes sanos. Además, ya que el incremento en el espesor íntima-media de la arteria carótida (un marcador subrogado de aterosclerosis) está directamente asociado con un mayor

riesgo de sufrir infarto agudo de miocardio e ictus [166], evaluamos la asociación entre PRDX-1 y TRX con este parámetro en sujetos asintomáticos. Nuestros resultados muestran una correlación positiva de PRDX-1 y TRX con el espesor de la íntima-media, que permaneció significativo tras el ajuste con factores clásicos de riesgo cardiovascular tales como hipertensión, diabetes o hipercolesterolemia. Es interesante comentar que se ha demostrado previamente que la actividad NADPH oxidasa fagocítica correlaciona con el grosor íntima-media carotídeo en pacientes asintomáticos de aterosclerosis [275]. Además, encontramos que los niveles plasmáticos de PRDX-1 y TRX correlacionan positivamente con la producción de superóxido dependiente de la NADPH oxidasa en monocitos de pacientes asintomáticos. De acuerdo con nuestros resultados, estudios previos han asociado los niveles circulantes de PRDX-1 y TRX con otros marcadores de estrés oxidativo (como por ejemplo la mieloperoxidasa o la 8-hydroxy-2'-deoxyguanosine) en diferentes patologías [51, 99]. Finalmente, mediante análisis de regresión demostramos una correlación positiva entre PRDX-1 y TRX en plasma tanto de pacientes carotídeos como de sujetos asintomáticos, lo que podría sugerir una respuesta coordinada de ambas proteínas al aumento en el estrés oxidativo presente en aterotrombosis. En consonancia, hemos descrito tanto a PRDX-1 como a TRX como nuevos biomarcadores de aneurisma aórtico abdominal (AAA)[145, 146]. El AAA es una forma específica de aterotrombosis, que se define por la progresiva pérdida en la capacidad de resistencia frente a la alta presión intraluminal, relacionada con la degradación de la pared arterial, dando lugar a la dilatación y rotura de esta [150]. En esos trabajos demostramos que tanto PRDX-1 como TRX correlacionan con el diámetro del AAA y su crecimiento [145, 146].

Por otro lado, se ha observado un aumento en la expresión de PRDX-1 en aortas de ApoE$^{-/-}$ con aterosclerosis avanzada, posiblemente como un mecanismo de respuesta al aumento en el estrés oxidativo [147]. Sin embargo, estos niveles elevados eran inferiores a los encontrados en ratones de fenotipo salvaje de la misma edad [147]. Por el contrario, los niveles proteicos de TRX en las placas de ateroma del ratón ApoE$^{-/-}$ aumentan en etapas tempranas, disminuyen de manera muy significativa según avanza el desarrollo de la lesión [42]. Esto podría indicar que en etapas iniciales las células del sistema vascular intentan responder mediante un aumento en los niveles de TRX, protegiéndose de esta forma frente al ambiente prooxidativo, sin embargo es posible que el desarrollo de la lesión en etapas más avanzadas se deba en parte al desequilibrio agudo en los mecanismos antioxidantes, entre los que se encontrarían los niveles disminuidos de TRX. Al contrario que el ratón que no tiene TRX, el ratón que no posee PRDX-1 es viable, con un fenotipo caracterizado por anemia hemolítica causada por un incremento en las ROS de los RBCs. Además PRDX-1 tiene propiedades supresoras de tumores ya que el ratón KO muestra una frecuencia incrementada que tumores múltiples malignos a medida que envejecen, lo que se

explica por la acumulación de tejido dañado debido al exceso de ROS [164]. Asimismo, PRDX-1 disminuye la adhesión leucocitaria al endotelio vascular y la activación del mismo, ya que modula la secreción mediada por ROS de los cuerpos de Weibel-Palade o la expresión de moléculas de adhesión tales como la p-selectina o el factor de von Willebrand [110]. El ratón doble KO para PRDX-1 y ApoE alimentado con dieta normal mostraba lesiones ateroscleróticas más grandes y más ricas en macrófagos que el ratón KO para ApoE [110]. Además, mediante la eliminación del H_2O_2, PRDX-1 puede inhibir la ruta de NF-ϰB y consecuentemente la respuesta inflamatoria [202]. Estos últimos estudios muestran lo que podría suponer un componente aditivo que explicaría el desarrollo de aterosclerosis en el ratón $ApoE^{-/-}$. En este trabajo nosotros ampliamos estos datos a placas de aterosclerosis carotídea humana, mostrando expresión de PRDX-1 y TRX en macrófagos de la placa, así como en CMLVs y eritrocitos. En este sentido, recientemente ha sido descrito que TRX se expresa en placas ateroscleróticas humanas asociada a la hemorragia intraplaca, posiblemente como una respuesta antioxidante de las células vasculares al medio prooxidativo que les rodea [167]. En placas de pacientes con aterosclerosis [169] y en arterias dañadas por el modelo de balón [239], la expresión de TRX tanto en el endotelio como en macrófagos se encuentra aumentada. Igualmente, numerosos miembros de la NADPH oxidasa fagocítica han sido descritos en placas ateroscleróticas humanas [116], entre ellos p22phox [13]. En este trabajo nosotros mostramos la colocalización de PRDX-1/TRX con p22phox en placas humanas de pacientes con aterosclerosis carotídea, lo que sugiere una asociación molecular entre la NADPH oxidasa y las proteínas PRDX-1/TRX en las células presentes en la lesión aterosclerótica, principalmente macrófagos y CMLVs. Para profundizar en la relación entre la NADPH oxidasa y PRDX-1/TRX, llevamos a cabo estudios *in vitro* en monocitos humanos estimulados con PMA, un conocido agente inductor de la NADPH oxidasa [276]. En estas condiciones experimentales, los niveles intracelulares de PRDX-1/TRX disminuyen, mientras que sus respectivos niveles extracelulares se incrementan (Figura 9). Este efecto se revirtió en presencia del inhibidor de la NADPH oxidasa, apocicina, lo que sugiere que los niveles de PRDX-1/TRX dependen de la producción de ROS dependiente de la actividad NADPH oxidasa. Asimismo, hallamos que este proceso de secreción puede producirse de manera activa mediante exosomas (Figura 9). La producción y el contenido de los exosomas puede verse modificada por la activación celular, como sugiere en el caso del PMA, y parece que puede verse revertido parcialmente con el uso de apocinina. Estas vesículas han sido implicadas en la patogénesis de la trombosis, diabetes, inflamación, aterosclerosis y la proliferación celular vascular [12]. Por otro lado, también demostramos que existe una liberación al medio extracelular no dependiente de exosomas (Figura 9). En el caso de la TRX, el tráfico intracelular puede ser llevado a cabo por la proteína TXNIP,

mientras que en el caso de la PRDX parece que su transporte hacia la membrana plasmática en respuesta al PMA [125] puede depender de su unión a PKC para la formación de vesículas de transporte como han demostrado otros autores [256]. Además, las modificaciones en los niveles intra y extracelulares de PRX-1/TRX parecen ser independientes de la síntesis *de novo*. De hecho, hallamos que tras la estimulación con PMA la célula respondería activando un mecanismo compensatorio para paliar la ausencia de PRX-1/TRX intracelular mediante el aumento en sus niveles de RNAm. Por otro lado, esta secreción de PRX-1/TRX al medio extracelular parece ser una respuesta coordinada celular a una situación de estrés oxidativo para intentar mantener la homeostasis celular y no una liberación derivada de la muerte celular por necrosis o apoptosis. Dicha respuesta se confirma a partir de los datos provenientes de la enzima MnSOD. La expresión de esta enzima puede verse aumentada por la exposición a estrés oxidativo [224] o ésteres de forbol, a través de un mecanismo dependiente de PKC [238, 260], tal y como nosotros demostramos en este trabajo tras la estimulación con PMA. Asimismo, otros autores han demostrado que la adición de TRX extracelular induce la expresión de MnSOD [43] lo que incidiría en la teoría de las "moonlighting proteins" ya que TRX actuaría en ese caso como una proteína transductora de señales. Sin embargo, aunque TRX podría estar implicada en la regulación de numerosos factores de transcripción [189] que podrían implicar la regulación de MnSOD, la modulación de los niveles de esta enzima podría estar causada por múltiples factores relativos al estado redox celular. En este sentido también hallamos que la preincubación con apocicina en los THP-1 aumenta de manera drástica los niveles de MnSOD. Estos datos sugieren que la apocinina disminuye el estrés oxidativo celular mediante diferentes mecanismos, que no se limitan exclusivamente a la inhibición de la actividad de la NAPDH oxidasa como demostramos en este trabajo, sino también a través del aumento de enzimas antioxidantes como la MnSOD. Sin embargo, los datos sobre la concentración intracelular de GSH muestran que la célula monocítica bajo estimulación con PMA disminuye los niveles intracelulares de GSH, aumentando los respectivos extracelulares. Tanto los niveles intracelulares de GSH como la actividad GSH peroxidasa están inversamente relacionados con la capacidad celular de oxidar LDLs en macrófagos [206]. Los sistemas TRX y el GSH regulan la actividad de numerosos factores de transcripción, cinasas y fosfatasas, estando implicados en el control del crecimiento y muerte celular, o en la señalización tanto en el interior como en el exterior de la célula [199, 58]. Los requerimientos redox de cada compartimento celular están dirigidos principalmente por la pareja formada por el GSH y el GSH oxidado [71]. De acuerdo con estos hallazgos, otros autores han demostrado la capacidad de diversos compuestos como la N-acetilcisteína y el difeniliodonio para reducir los niveles de TRX inducidos por H_2O_2 o por homocisteína en células vasculares

humanas [42, 112]. Además también estudiamos el estado oxidado de PRX y demostramos que esta proteína se encuentra altamente oxidada bajo estimulación con PMA, lo que inactivaría su función peroxidasa y podría dar lugar a la formación de oligómeros, inhibiendo de esta forma su función protectora frente a la producción escesiva de ROS [95]. Para confirmar la especificad de la implicación de la NADPH oxidasa y descartar un efecto general de la apocicina en el transporte de PRDX y TRX hacia el exterior celular, se llevaron a cabo nuevos estudios en los que se silenció la subunidad catalítica de la NADPH en macrófagos NOX2. El silenciamiento de NOX2 revirtió la disminución intracelular de los niveles de PRDX-1/TRX inducidos por PMA. En conjunto, todos estos datos apoyan la hipótesis de que el estrés oxidativo induce la liberación de PRDX-1/TRX y sugiere la potencial implicación de la producción de superóxido dependiente de la NADPH oxidasa y el incremento de los niveles de PRDX-1/TRX en aterotrombosis (Figura 9). Sin embargo, y dado que en los estudios humanos no proporcionamos evidencias directas del posible papel de la NADPH oxidasa vascular en aterosclerosis, es importante puntualizar que el incremento de la NADPH oxidasa fagocítica no tiene por qué ser necesariamente el único factor responsable para el incremento en los niveles de PRDX-1/TRX. Además, hay que tener en cuenta la participación de otras que en la generación de ROS en aterosclerosis, entre ellas la lipooxigenasa, la xantina oxidasa y la sintasa de NO [139]. Es interesante resaltar que los efectos pleiotrópicos de las estatinas, usadas como agentes cardioprotectores, dependen de la actividad de eNOS [54, 124, 262]. El NO incrementa la S-nitrosilación de TRX estimulando su actividad lo que resulta finalmente en una reducción en la producción de ROS en ECs [70]. Por otro lado, se ha demostrado que PRDX-1 controla la proliferación de CMLVs [103] y suprime la formación de la lesión aterosclerótica en el ratón ApoE$^{-/-}$, probablemente debido a su actividad anti-inflamatoria [110]. De manera similar, TRX podría actuar como una proteína anti-inflamatoria, ya que la TRX extracelular reduce la expresión de la IL-1β por los monocitos /macrófagos en condiciones pro-inflamatorias [23]. La TRX circulante también actúa como un quimioatractante para monocitos, neutrófilos y linfocitos [21], e inhibe la migración de los neutrófilos tanto *in vitro* como *in vivo* [160]. De acuerdo con estos datos, TRX podría ser una posible diana terapéutica para el tratamiento de las enfermedades del sistema vascular. Algunos estudios en modelos animales confirman este punto, por ejemplo la administración de TRX recombinante previene la miocarditis inducida por miosina [134] y el enfisema inducido por tabaco [212]. Por tanto, se podría hipotetizar que los altos niveles extracelulares de PRDX-1/TRX observados en pacientes de aterotrombosis podrían reflejar un mecanismo inhibitorio de reclutamiento de leucocitos hacia la placa. Sin embargo, son necesarios más estudios en modelos animales para apoyar esta hipótesis.

En resumen, hemos demostrado que los niveles circulantes de PRDX-1/TRX están elevados en pacientes con aterosclerosis carotídea y correlacionan con el espesor íntima-media carotídeo y la producción de superóxido dependiente de la NADPH oxidasa en pacientes asintomáticos, apoyando la utilidad potencial de estas moléculas como biomarcadores de estrés oxidativo en aterosclerosis.

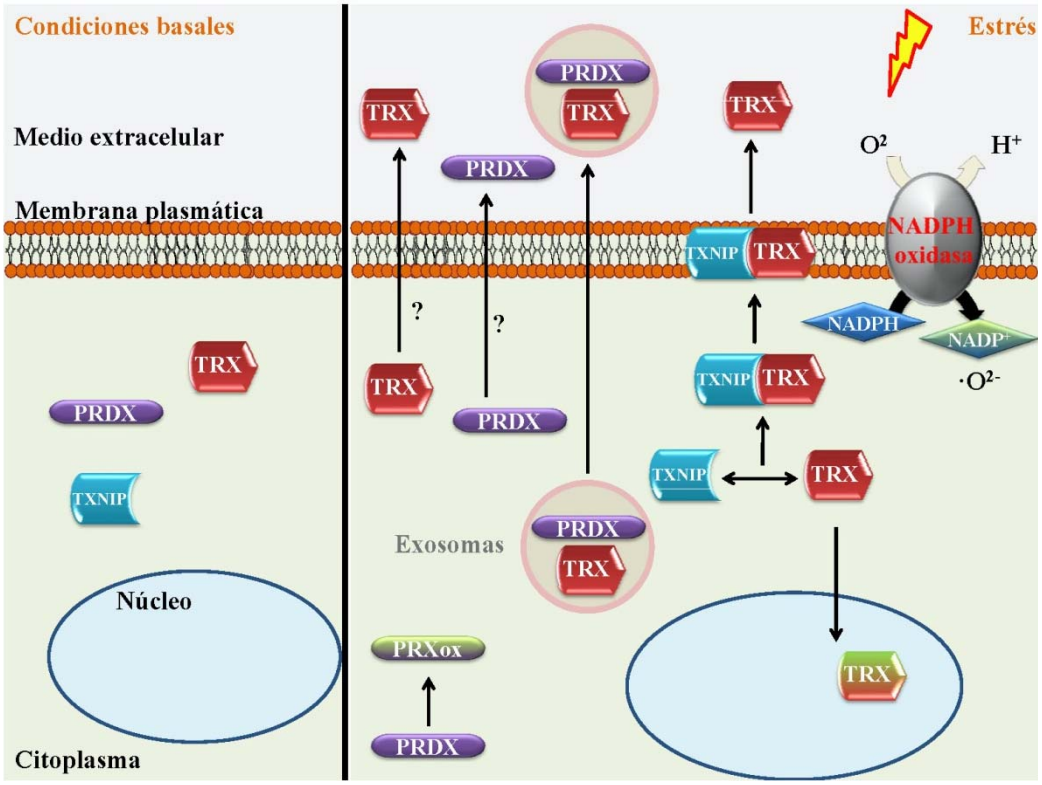


Figura 9.- Esquema de las modificaciones intracelulares y en el transporte bajo estrés oxidativo de PRDX-1/TRX.

Las **funciones intracelulares de las CSPs** son clave, ya que muchas de ellas protegen a las células de la vasculatura frente a estímulos nocivos. Por otro lado, la expresión de las CSPs es modulada por numerosos estímulos implicados en la aterogénesis incluyendo inflamación, estrés oxidativo, proteólisis o apoptosis. Esto podría reflejar cambios en los niveles de **CSPs en el medio extracelular**, pudiéndose manifestar en los niveles plasmáticos. Sin embargo, aunque en las enfermedades cardiovasculares se ha sugerido a numerosas HSPs como marcadores de la patología asociadas al desarrollo de estas enfermedades, hay que ser cautos al analizar estos datos ya que su expresión puede ser transitoria en respuesta a eventos agudos como el infarto de miocardio, o reflejar un estado crónico de desequilibrio redox o inflamatorio más que un riesgo específico. Como consecuencia, los esfuerzos terapéuticos para el tratamiento de enfermedades

cardiovasculares como la aterosclerosis deberían focalizarse no sólo en la regulación de los niveles intracelulares y extracelulares de las CSPs, sino también el reconocimiento de las CSPs por sus receptores y su posterior internalización. Así finalmente, las CSPs podrían ser consideradas como dianas potenciales para el fortalecimiento de las defensas vasculares y retrasar o evitar las complicaciones clínicas asociadas a la aterotrombosis.

Líneas de trabajo abiertas tras la realización de esta tesis

Aunque este trabajo ha conseguido responder numerosas preguntas planteadas al inicio de la tesis, también ha originado muchas más preguntas, abriendo nuevas líneas de trabajo para intentar responderlas. Para ello estamos realizando estudios *in vitro* usando un péptido que inhibe específicamente la actividad chaperona de HSP70. Además, intentaremos profundizar a nivel molecular sobre las acciones que provocan ambas aproximaciones terapéuticas (17-DMAG, péptido inhibidor de HSP70) en sus proteínas cliente. Por otro lado, para discernir del efecto inhibitorio en la actividad ATPasa del 17-DMAG al unirse HSP90, del efecto pleiotrópico que da lugar a la sobreexpresión de HSPs protectoras (HSP27 y HSP70), estamos realizando ensayos preliminares *in vitro* con adenovirus que consiguen la sobreexpresión de HSF1. De esta forma es posible conseguir el aumento en los niveles de HSPs protectoras sin inhibir la actividad de HSP90. Conjuntamente, para abordar el estudio del papel de las HSPs a nivel extracelular estamos intentando desarrollar una proteína recombinante de HSP70, bien con secuencias que le permiten penetrar en las células, bien con la ausencia de las mismas. De esta forma podríamos estudiar el papel extracelular de esta HSP y además estudiar el posible papel terapéutico de la administración de la misma.

Finalmente, una vez comprobado en esta tesis el papel citoprotector de las proteínas de la familia de la TRX (PRDX-1/TRX) frente al estrés oxidativo en aterosclerosis, estamos poniendo en marcha un estudio *in vivo* en el que estudiaremos la modulación terapéutica de estas proteínas y su efecto en el modelo de aterosclerosis de ApoE$^{-/-}$, tanto en la progresión como en la regresión.

Estas cuatro nuevas aproximaciones buscan diseccionar y profundizar en los efectos individualizados y la función en aterogénesis de cada CSP, lo que facilitaría el desarrollo de nuevas terapias en aterosclerosis o la mejora de las ya existentes.

Líneas de pensamiento abiertas durante la realización de esta tesis

Diversos procesos que intervienen en la patogenia de la aterosclerosis afectan directamente a la estabilidad de las proteínas. El principio operativo de la PN [185], entendiendo a la PN como un programa global de mantenimiento del correcto ensamblaje proteico que podría

usarse para desarrollar mejores herramientas para mejorar la salud en humanos, sugiere que es necesario solventar los problemas en la homeostasis proteica de manera general a través de la modulación del PN, efecto que se consigue al usar el inhibidor específico de la HSP90, 17-DMAG. La explicación de por qué los inhibidores de HSP90 promueven la degradación de proteínas vía ubiquitinación-proteosoma [259, 219] se debe a que la unión de HSP90 a su proteína cliente inhabilita su degradación, e inhibidores como el 17-DMAG impiden esa unión. Esta observación de la maquinaria de las chaperonas se complementa con el efecto opuesto de ambas proteínas: HSP70 aumentando la degradación de proteínas cliente mediante ubiquitación y HSP90 inhibiéndola, siendo esta la que toma la decisión sobre el destino de una proteína desensamblada en un escenario de estrés[190]. Además, el estado redox celular afecta de manera clave al mantenimiento de la proteostass mediante la modificación de la conformación de las proteínas (PRX-1/TRX), altera las interacciones proteína-proteína (TXNIP-TRX) y/o promueve el tráfico intracelular de proteínas de respuesta al estrés (liberación de PRX-1/TRX). Todos estos datos muestran la importancia del desarrollo de nuevas terapias y estrategias de prevención que modulen las disfunciones vasculares asociadas a las alteraciones proteicas y el desequilibrio redox celular. La visión de la importancia de la PN en la fisiología de los organismos está minusvalorada, sin embargo cada vez parece ser más evidente que enfermedades como la aterosclerosis podrían beneficiarse de un mejor conocimiento global del funcionamiento de la PN. Sin embargo, el estudio molecular de la funcionalidad chaperona de las CSPs sobre sus proteínas cliente y la importancia de la proteostasis en aterosclerosis no han sido profundamente estudiados en este trabajo y futuros estudios moleculares en este campo podrían arrojar luz sobre estos procesos complejos implicados en el desarrollo de la placa de ateroma.

V. CONCLUSIONES

1. HSP90 se encuentra sobre-expresada en la placa aterosclerótica, sobre todo en las placas más vulnerables, por lo que podría considerarse como un factor asociado a la inestabilidad de la misma, mientras que HSP70 podría relacionarse con la estabilidad de la placa de ateroma.

2. Los inhibidores de HSP90, como el 17-AAG y el 17-DMAG, poseen efectos pleiotrópicos entre los que se encuentran la modulación de numerosos mediadores inflamatorios implicados en aterosclerosis y la sobreexpresión de HSPs protectoras, como la HSP70.

3. El 17-DMAG interfiere con el desarrollo de la placa y reduce el contenido lipídico de la misma, pudiendo promover la estabilidad de la placa a través de la reducción de factores inflamatorios e infiltración de monocitos/macrófagos.

4. La inhibición de HSP90 es capaz de disminuir el estrés oxidativo y la transición monocito/macrófago en aterosclerosis experimental, apuntando hacia un posible uso terapéutico del 17-DMAG en enfermedades relacionadas con el estrés oxidativo y la inflamación.

5. El incremento en los niveles de las subunidades de la NADPH oxidasa, NOX1 y NOXO1, durante el proceso de diferenciación de los monocitos y en las placas vulnerables de aterosclerosis avanzada podrían ser marcadores de inestabilidad de lesión ateromatosa.

6. La liberación al medio extracelular de PRDX-1/TRX está asociada a la producción de ROS dependiente de la NAPDH oxidasa, un proceso mediado, al menos en parte, por exosomas.

7. Los niveles circulantes de PRDX-1/TRX están elevados en pacientes con aterosclerosis carotídea en comparación son sujetos sanos.

8. Asimismo, los niveles circulantes de PRDX-1/TRX correlacionan con el grosor íntima-media carotídeo y la producción de superóxido dependiente de la NADPH oxidasa en pacientes asintomáticos, apoyando la utilidad potencial de estas moléculas como biomarcadores de estrés oxidativo en aterosclerosis.

Como conclusión personal y global de esta trabajo, quiero resaltar como mensaje final que las proteínas de respuesta al estrés son algo más que chaperonas, ya que ocupan un espacio funcional continuo que abarca tanto su ampliamente descrito papel efector en el

ensamblaje/desensamblaje y degradación de proteínas, como sus escasamente descritos hasta el momento, efectos en la señalización intracelular y como comunicadores intercelulares.

VI. BIBLIOGRAFÍA

(1) Adhikari N, Charles N, Lehmann U, Hall JL. Transcription factor and kinase-mediated signaling in atherosclerosis and vascular injury. *Curr Atheroscler Rep* 2006 May;8(3):252-60.

(2) Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, Neve EP, Scheynius A, Gabrielsson S. Exosomes with immune modulatory features are present in human breast milk. *J Immunol* 2007 August 1;179(3):1969-78.

(3) Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, Iida M. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation* 2004 January 20;109(2):227-33.

(4) Aikawa M, Libby P. The vulnerable atherosclerotic plaque: pathogenesis and therapeutic approach. *Cardiovasc Pathol* 2004 May;13(3):125-38.

(5) Akerfelt M, Morimoto RI, Sistonen L. Heat shock factors: integrators of cell stress, development and lifespan. *Nat Rev Mol Cell Biol* 2010 August;11(8):545-55.

(6) Albertini R, Moratti R, De LG. Oxidation of low-density lipoprotein in atherosclerosis from basic biochemistry to clinical studies. *Curr Mol Med* 2002 September;2(6):579-92.

(7) Ali A, Bharadwaj S, O'Carroll R, Ovsenek N. HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. *Mol Cell Biol* 1998 September;18(9):4949-60.

(8) Ananthan J, Goldberg AL, Voellmy R. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* 1986 April 25;232(4749):522-4.

(9) Ananthan J, Goldberg AL, Voellmy R. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* 1986 April 25;232(4749):522-4.

(10) Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002 April 26;277(17):15028-34.

(11) Ashburner M. Patterns of puffing activity in the salivary gland chromosomes of Drosophila. V. Responses to environmental treatments. *Chromosoma* 1970;31(3):356-76.

(12) Azevedo LC, Pedro MA, Laurindo FR. Circulating microparticles as therapeutic targets in cardiovascular diseases. *Recent Pat Cardiovasc Drug Discov* 2007 January;2(1):41-51.

(13) Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M. Expression of NADH/NADPH oxidase p22phox in human coronary arteries. *Circulation* 1999 October 5;100(14):1494-8.

(14) Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. *Arch Biochem Biophys* 2002 January 15;397(2):342-4.

(15) Baldwin AS, Jr. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001 January;107(1):3-6.

(16) Baler R, Dahl G, Voellmy R. Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Mol Cell Biol* 1993 April;13(4):2486-96.

(17) Ballatori N, Krance SM, Marchan R, Hammond CL. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. *Mol Aspects Med* 2009 February;30(1-2):13-28.

(18) Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997 April 10;336(15):1066-71.

(19) Barranco-Medina S, Lazaro JJ, Dietz KJ. The oligomeric conformation of peroxiredoxins links redox state to function. *FEBS Lett* 2009 June 18;583(12):1809-16.

(20) Bentzon JF, Falk E. Atherosclerotic lesions in mouse and man: is it the same disease? *Curr Opin Lipidol* 2010 October;21(5):434-40.

(21) Bertini R, Howard OM, Dong HF, Oppenheim JJ, Bizzarri C, Sergi R, Caselli G, Pagliei S, Romines B, Wilshire JA, Mengozzi M, Nakamura H, Yodoi J, Pekkari K, Gurunath R, Holmgren A, Herzenberg LA, Herzenberg LA, Ghezzi P. Thioredoxin, a redox enzyme released in infection and inflammation, is a unique chemoattractant for neutrophils, monocytes, and T cells. *J Exp Med* 1999 June 7;189(11):1783-9.

(22) Biamonte MA, Van de Water R, Arndt JW, Scannevin RH, Perret D, Lee WC. Heat shock protein 90: inhibitors in clinical trials. *J Med Chem* 2010 January 14;53(1):3-17.

(23) Billiet L, Furman C, Larigauderie G, Copin C, Brand K, Fruchart JC, Rouis M. Extracellular human thioredoxin-1 inhibits lipopolysaccharide-induced interleukin-1beta expression in human monocyte-derived macrophages. *J Biol Chem* 2005 December 2;280(48):40310-8.

(24) Blanco-Colio LM, Tunon J, Martin-Ventura JL, Egido J. Anti-inflammatory and immunomodulatory effects of statins. *Kidney Int* 2003 January;63(1):12-23.

(25) Blanco-Colio LM, Valderrama M, Alvarez-Sala LA, Bustos C, Ortego M, Hernandez-Presa MA, Cancelas P, Gomez-Gerique J, Millan J, Egido J. Red wine intake prevents nuclear factor-kappaB activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia. *Circulation* 2000 August 29;102(9):1020-6.

(26) Blumberg J. Use of biomarkers of oxidative stress in research studies. *J Nutr* 2004 November;134(11):3188S-9S.

(27) Broadley SA, Hartl FU. The role of molecular chaperones in human misfolding diseases. *FEBS Lett* 2009 August 20;583(16):2647-53.

(28) Buschow SI, van Balkom BW, Aalberts M, Heck AJ, Wauben M, Stoorvogel W. MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. *Immunol Cell Biol* 2010 November;88(8):851-6.

(29) Businaro R, Profumo E, Tagliani A, Buttari B, Leone S, D'Amati G, Ippoliti F, Leopizzi M, D'Arcangelo D, Capoano R, Fumagalli L, Salvati B, Rigano R. Heat-shock protein 90: a novel autoantigen in human carotid atherosclerosis. *Atherosclerosis* 2009 November;207(1):74-83.

(30) Carr SC, Farb A, Pearce WH, Virmani R, Yao JS. Activated inflammatory cells are associated with plaque rupture in carotid artery stenosis. *Surgery* 1997 October;122(4):757-63.

(31) Cha MK, Kim IH. Thioredoxin-linked peroxidase from human red blood cell: evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cell. *Biochem Biophys Res Commun* 1995 December 26;217(3):900-7.

(32) Chae HZ, Kim HJ, Kang SW, Rhee SG. Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Res Clin Pract* 1999 September;45(2-3):101-12.

(33) Chang JW, Lee SH, Jeong JY, Chae HZ, Kim YC, Park ZY, Yoo YJ. Peroxiredoxin-I is an autoimmunogenic tumor antigen in non-small cell lung cancer. *FEBS Lett* 2005 May 23;579(13):2873-7.

(34) Chang JW, Lee SH, Lu Y, Yoo YJ. Transforming growth factor-beta1 induces the non-classical secretion of peroxiredoxin-I in A549 cells. *Biochem Biophys Res Commun* 2006 June 23;345(1):118-23.

(35) Chatterjee A, Dimitropoulou C, Drakopanayiotakis F, Antonova G, Snead C, Cannon J, Venema RC, Catravas JD. Heat shock protein 90 inhibitors prolong survival, attenuate inflammation, and reduce lung injury in murine sepsis. *Am J Respir Crit Care Med* 2007 October 1;176(7):667-75.

(36) Chen F, Pandey D, Chadli A, Chen T, Catravas JD, Fulton D. Hsp90 regulates NADPH oxidase activity and is necessary for superoxide but not hydrogen peroxide production. *Antioxid Redox Signal* 2011 January 2.

(37) Chen Y, Voegeli TS, Liu PP, Noble EG, Currie RW. Heat shock paradox and a new role of heat shock proteins and their receptors as anti-inflammation targets. *Inflamm Allergy Drug Targets* 2007 June;6(2):91-100.

(38) Cheng G, Lambeth JD. NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *J Biol Chem* 2004 February 6;279(6):4737-42.

(39) Choi DS, Lee JM, Park GW, Lim HW, Bang JY, Kim YK, Kwon KH, Kwon HJ, Kim KP, Gho YS. Proteomic analysis of microvesicles derived from human colorectal cancer cells. *J Proteome Res* 2007 December;6(12):4646-55.

(40) Choi MH, Lee IK, Kim GW, Kim BU, Han YH, Yu DY, Park HS, Kim KY, Lee JS, Choi C, Bae YS, Lee BI, Rhee SG, Kang SW. Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* 2005 May 19;435(7040):347-53.

(41) Cuaz-Perolin C, Billiet L, Bauge E, Copin C, Scott-Algara D, Genze F, Buchele B, Syrovets T, Simmet T, Rouis M. Antiinflammatory and antiatherogenic effects of the NF-kappaB inhibitor acetyl-11-keto-beta-boswellic acid in LPS-challenged ApoE-/- mice. *Arterioscler Thromb Vasc Biol* 2008 February;28(2):272-7.

(42) Dai J, Wang X, Feng J, Kong W, Xu Q, Shen X, Wang X. Regulatory role of thioredoxin in homocysteine-induced monocyte chemoattractant protein-1 secretion in monocytes/macrophages. *FEBS Lett* 2008 November 26;582(28):3893-8.

(43) Das KC, Lewis-Molock Y, White CW. Elevation of manganese superoxide dismutase gene expression by thioredoxin. *Am J Respir Cell Mol Biol* 1997 December;17(6):713-26.

(44) Dello RC, Polak PE, Mercado PR, Spagnolo A, Sharp A, Murphy P, Kamal A, Burrows FJ, Fritz LC, Feinstein DL. The heat-shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin suppresses glial inflammatory responses and ameliorates experimental autoimmune encephalomyelitis. *J Neurochem* 2006 December;99(5):1351-62.

(45) Dessein AJ, Lenzi HL, Bina JC, Carvalho EM, Weiser WY, Andrade ZA, David JR. Modulation of eosinophil cytotoxicity by blood mononuclear cells from healthy subjects and patients with chronic schistosomiasis mansoni. *Cell Immunol* 1984 April 15;85(1):100-13.

(46) Didelot C, Lanneau D, Brunet M, Joly AL, De TA, Chiosis G, Garrido C. Anti-cancer therapeutic approaches based on intracellular and extracellular heat shock proteins. *Curr Med Chem* 2007;14(27):2839-47.

(47) Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HH, Harrison DG, Griendling KK. Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free Radic Biol Med* 2008 November 1;45(9):1340-51.

(48) Dikalova A, Clempus R, Lassegue B, Cheng G, McCoy J, Dikalov S, San MA, Lyle A, Weber DS, Weiss D, Taylor WR, Schmidt HH, Owens GK, Lambeth JD, Griendling KK. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. *Circulation* 2005 October 25;112(17):2668-76.

(49) Drummond GR, Selemidis S, Griendling KK, Sobey CG. Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat Rev Drug Discov* 2011 June;10(6):453-71.

(50) Dutta S, Rittinger K. Regulation of NOXO1 activity through reversible interactions with p22 and NOXA1. *PLoS One* 2010;5(5):e10478.

(51) Dworakowski R, Anilkumar N, Zhang M, Shah AM. Redox signalling involving NADPH oxidase-derived reactive oxygen species. *Biochem Soc Trans* 2006 November;34(Pt 5):960-4.

(52) Ellmark SH, Dusting GJ, Fui MN, Guzzo-Pernell N, Drummond GR. The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc Res* 2005 February 1;65(2):495-504.

(53) Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem* 1993 June 5;268(16):11811-6.

(54) Endres M, Laufs U, Huang Z, Nakamura T, Huang P, Moskowitz MA, Liao JK. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 1998 July 21;95(15):8880-5.

(55) Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation* 1995 August 1;92(3):657-71.

(56) Farb A, Burke AP, Tang AL, Liang TY, Mannan P, Smialek J, Virmani R. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. *Circulation* 1996 April 1;93(7):1354-63.

(57) Fenyo IM, Florea IC, Raicu M, Manea A. Tyrphostin AG490 reduces NAPDH oxidase activity and expression in the aorta of hypercholesterolemic apolipoprotein E-deficient mice. *Vascul Pharmacol* 2011 March 30.

(58) Finkel T. Redox-dependent signal transduction. *FEBS Lett* 2000 June 30;476(1-2):52-4.

(59) Fortuno A, Olivan S, Beloqui O, San JG, Moreno MU, Diez J, Zalba G. Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension. *J Hypertens* 2004 November;22(11):2169-75.

(60) Franco R, Schoneveld OJ, Pappa A, Panayiotidis MI. The central role of glutathione in the pathophysiology of human diseases. *Arch Physiol Biochem* 2007 October;113(4-5):234-58.

(61) Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 1995;64:97-112.

(62) Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. *Cell Cycle* 2006 November;5(22):2592-601.

(63) Geiszt M, Lekstrom K, Witta J, Leto TL. Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *J Biol Chem* 2003 May 30;278(22):20006-12.

(64) Gharavi NM, Alva JA, Mouillesseaux KP, Lai C, Yeh M, Yeung W, Johnson J, Szeto WL, Hong L, Fishbein M, Wei L, Pfeffer LM, Berliner JA. Role of the Jak/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids in vitro and in atherosclerosis in vivo. *J Biol Chem* 2007 October 26;282(43):31460-8.

(65) Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, Wang NS, Knepper MA. Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol* 2009 February;20(2):363-79.

(66) Gonzalez-Begne M, Lu B, Han X, Hagen FK, Hand AR, Melvin JE, Yates JR. Proteomic analysis of human parotid gland exosomes by multidimensional protein identification technology (MudPIT). *J Proteome Res* 2009 March;8(3):1304-14.

(67) Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* 2003 October 21;108(16):1912-6.

(68) Guijarro C, Egido J. Transcription factor-kappa B (NF-kappa B) and renal disease. *Kidney Int* 2001 February;59(2):415-24.

(69) Hackl C, Mori A, Moser C, Lang SA, Dayoub R, Weiss TS, Schlitt HJ, Geissler EK, Hellerbrand C, Stoeltzing O. Effect of heat-shock protein-90 (HSP90) inhibition on

human hepatocytes and on liver regeneration in experimental models. *Surgery* 2010 May;147(5):704-12.

(70) Haendeler J, Hoffmann J, Zeiher AM, Dimmeler S. Antioxidant effects of statins via S-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. *Circulation* 2004 August 17;110(7):856-61.

(71) Hansen JM, Go YM, Jones DP. Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annu Rev Pharmacol Toxicol* 2006;46:215-34.

(72) Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005 April 21;352(16):1685-95.

(73) Hansson GK. Regulation of immune mechanisms in atherosclerosis. *Ann N Y Acad Sci* 2001 December;947:157-65.

(74) Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* 2003 February 6;91(3A):7A-11A.

(75) Hattori I, Takagi Y, Nakamura H, Nozaki K, Bai J, Kondo N, Sugino T, Nishimura M, Hashimoto N, Yodoi J. Intravenous administration of thioredoxin decreases brain damage following transient focal cerebral ischemia in mice. *Antioxid Redox Signal* 2004 February;6(1):81-7.

(76) Hellings WE, Peeters W, Moll FL, Piers SR, van SJ, Van der Spek PJ, de Vries JP, Seldenrijk KA, De Bruin PC, Vink A, Velema E, de Kleijn DP, Pasterkamp G. Composition of carotid atherosclerotic plaque is associated with cardiovascular outcome: a prognostic study. *Circulation* 2010 May 4;121(17):1941-50.

(77) Hemmingsen SM, Woolford C, van der Vies SM, Tilly K, Dennis DT, Georgopoulos CP, Hendrix RW, Ellis RJ. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* 1988 May 26;333(6171):330-4.

(78) Herbst M, Wanker EE. Small molecule inducers of heat-shock response reduce polyQ-mediated huntingtin aggregation. A possible therapeutic strategy. *Neurodegener Dis* 2007;4(2-3):254-60.

(79) Hess A, Wijayanti N, Neuschafer-Rube AP, Katz N, Kietzmann T, Immenschuh S. Phorbol ester-dependent activation of peroxiredoxin I gene expression via a protein kinase C, Ras, p38 mitogen-activated protein kinase signaling pathway. *J Biol Chem* 2003 November 14;278(46):45419-34.

(80) Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004 April;24(4):677-83.

(81) Hjerpe C, Johansson D, Hermansson A, Hansson GK, Zhou X. Dendritic cells pulsed with malondialdehyde modified low density lipoprotein aggravate atherosclerosis in Apoe(-/-) mice. *Atherosclerosis* 2010 April;209(2):436-41.

(82) Ho AW, Wong CK, Lam CW. Tumor necrosis factor-alpha up-regulates the expression of CCL2 and adhesion molecules of human proximal tubular epithelial cells through MAPK signaling pathways. *Immunobiology* 2008;213(7):533-44.

(83) Hohfeld J, Minami Y, Hartl FU. Hip, a novel cochaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. *Cell* 1995 November 17;83(4):589-98.

(84) Hokamaki J, Kawano H, Soejima H, Miyamoto S, Kajiwara I, Kojima S, Sakamoto T, Sugiyama S, Yoshimura M, Nakamura H, Yodoi J, Ogawa H. Plasma thioredoxin levels in patients with unstable angina. *Int J Cardiol* 2005 March 18;99(2):225-31.

(85) Holmgren A. Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxid Redox Signal* 2000;2(4):811-20.

(86) Hostein I, Robertson D, DiStefano F, Workman P, Clarke PA. Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. *Cancer Res* 2001 May 15;61(10):4003-9.

(87) House SD, Guidon PT, Jr., Perdrizet GA, Rewinski M, Kyriakos R, Bockman RS, Mistry T, Gallagher RA, Hightower LE. Effects of heat shock, stannous chloride, and gallium nitrate on the rat inflammatory response. *Cell Stress Chaperones* 2001 April;6(2):164-71.

(88) Hu W, Polinsky P, Sadoun E, Rosenfeld ME, Schwartz SM. Atherosclerotic lesions in the common coronary arteries of ApoE knockout mice. *Cardiovasc Pathol* 2005 May;14(3):120-5.

(89) Hua H, Munk S, Goldberg H, Fantus IG, Whiteside CI. High glucose-suppressed endothelin-1 Ca2+ signaling via NADPH oxidase and diacylglycerol-sensitive protein kinase C isozymes in mesangial cells. *J Biol Chem* 2003 September 5;278(36):33951-62.

(90) Hunter-Lavin C, Davies EL, Bacelar MM, Marshall MJ, Andrew SM, Williams JH. Hsp70 release from peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 2004 November 12;324(2):511-7.

(91) Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003 January;9(1):61-7.

(92) Hutchison KA, Stancato LF, Owens-Grillo JK, Johnson JL, Krishna P, Toft DO, Pratt WB. The 23-kDa acidic protein in reticulocyte lysate is the weakly bound component of the hsp foldosome that is required for assembly of the glucocorticoid receptor into a functional heterocomplex with hsp90. *J Biol Chem* 1995 August 11;270(32):18841-7.

(93) Hutt D, Balch WE. Cell Biology. The proteome in balance. *Science* 2010 August 13;329(5993):766-7.

(94) Inoue S, Egashira K, Ni W, Kitamoto S, Usui M, Otani K, Ishibashi M, Hiasa K, Nishida K, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 2002 November 19;106(21):2700-6.

(95) Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong GW, Yun DJ, Rhee SG, Cho MJ, Lee SY. Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* 2004 May 28;117(5):625-35.

(96) Jaquet V, Marcoux J, Forest E, Leidal KG, McCormick S, Westermaier Y, Perozzo R, Plastre O, Fioraso-Cartier L, Diebold B, Scapozza L, Nauseef WM, Fieschi F, Krause KH, Bedard K. NADPH oxidase (NOX) isoforms are inhibited by celastrol with a dual mode of action. *Br J Pharmacol* 2011 September;164(2b):507-20.

(97) Jeffery CJ. Moonlighting proteins. *Trends Biochem Sci* 1999 January;24(1):8-11.

(98) Jeziorska M, Woolley DE. Local neovascularization and cellular composition within vulnerable regions of atherosclerotic plaques of human carotid arteries. *J Pathol* 1999 June;188(2):189-96.

(99) Jikimoto T, Nishikubo Y, Koshiba M, Kanagawa S, Morinobu S, Morinobu A, Saura R, Mizuno K, Kondo S, Toyokuni S, Nakamura H, Yodoi J, Kumagai S. Thioredoxin as a biomarker for oxidative stress in patients with rheumatoid arthritis. *Mol Immunol* 2002 February;38(10):765-72.

(100) Jo H, Song H, Mowbray A. Role of NADPH oxidases in disturbed flow- and. *Antioxid Redox Signal* 2006 September;8(9-10):1609-19.

(101) Jolly C, Morimoto RI. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J Natl Cancer Inst* 2000 October 4;92(19):1564-72.

(102) Jones DP. Redefining oxidative stress. *Antioxid Redox Signal* 2006 September;8(9-10):1865-79.

(103) Jones FS, Meech R, Edelman DB, Oakey RJ, Jones PL. Prx1 controls vascular smooth muscle cell proliferation and tenascin-C expression and is upregulated with Prx2 in pulmonary vascular disease. *Circ Res* 2001 July 20;89(2):131-8.

(104) Junn E, Han SH, Im JY, Yang Y, Cho EW, Um HD, Kim DK, Lee KW, Han PL, Rhee SG, Choi I. Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol* 2000 June 15;164(12):6287-95.

(105) Kaartinen M, Penttila A, Kovanen PT. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* 1994 October;90(4):1669-78.

(106) Kamizato M, Nishida K, Masuda K, Takeo K, Yamamoto Y, Kawai T, Teshima-Kondo S, Tanahashi T, Rokutan K. Interleukin 10 inhibits interferon gamma- and tumor necrosis factor alpha-stimulated activation of NADPH oxidase 1 in human colonic epithelial cells and the mouse colon. *J Gastroenterol* 2009;44(12):1172-84.

(107) Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE. Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 2009 January;14(1):105-11.

(108) Kim JM, Kim H, Kwon SB, Lee SY, Chung SC, Jeong DW, Min BM. Intracellular glutathione status regulates mouse bone marrow monocyte-derived macrophage differentiation and phagocytic activity. *Biochem Biophys Res Commun* 2004 December 3;325(1):101-8.

(109) Kim JY, Kim WJ, Kim H, Suk K, Lee WH. The Stimulation of CD147 Induces MMP-9 Expression through ERK and NF-kappaB in Macrophages: Implication for Atherosclerosis. *Immune Netw* 2009 June;9(3):90-7.

(110) Kisucka J, Chauhan AK, Patten IS, Yesilaltay A, Neumann C, Van Etten RA, Krieger M, Wagner DD. Peroxiredoxin1 prevents excessive endothelial activation and early atherosclerosis. *Circ Res* 2008 September 12;103(6):598-605.

(111) Ko HS, Bailey R, Smith WW, Liu Z, Shin JH, Lee YI, Zhang YJ, Jiang H, Ross CA, Moore DJ, Patterson C, Petrucelli L, Dawson TM, Dawson VL. CHIP regulates leucine-rich repeat kinase-2 ubiquitination, degradation, and toxicity. *Proc Natl Acad Sci U S A* 2009 February 24;106(8):2897-902.

(112) Kondo N, Ishii Y, Kwon YW, Tanito M, Horita H, Nishinaka Y, Nakamura H, Yodoi J. Redox-sensing release of human thioredoxin from T lymphocytes with negative feedback loops. *J Immunol* 2004 January 1;172(1):442-8.

(113) Kregel KC, Zhang HJ. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *Am J Physiol Regul Integr Comp Physiol* 2007 January;292(1):R18-R36.

(114) Kummar S, Gutierrez ME, Gardner ER, Chen X, Figg WD, Zajac-Kaye M, Chen M, Steinberg SM, Muir CA, Yancey MA, Horneffer YR, Juwara L, Melillo G, Ivy SP, Merino M, Neckers L, Steeg PS, Conley BA, Giaccone G, Doroshow JH, Murgo AJ. Phase I trial of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), a heat shock protein inhibitor, administered twice weekly in patients with advanced malignancies. *Eur J Cancer* 2010 January;46(2):340-7.

(115) Lancet JE, Gojo I, Burton M, Quinn M, Tighe SM, Kersey K, Zhong Z, Albitar MX, Bhalla K, Hannah AL, Baer MR. Phase I study of the heat shock protein 90 inhibitor alvespimycin (KOS-1022, 17-DMAG) administered intravenously twice weekly to patients with acute myeloid leukemia. *Leukemia* 2010 April;24(4):699-705.

(116) Lassegue B, Griendling KK. NADPH oxidases: functions and pathologies in the vasculature. *Arterioscler Thromb Vasc Biol* 2010 April;30(4):653-61.

(117) Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001 May 11;88(9):888-94.

(118) Latchman DS. Transcription factors: an overview. *Int J Biochem Cell Biol* 1997 December;29(12):1305-12.

(119) Lavrovsky Y, Chatterjee B, Clark RA, Roy AK. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp Gerontol* 2000 August;35(5):521-32.

(120) Leavey PJ, Gonzalez-Aller C, Thurman G, Kleinberg M, Rinckel L, Ambruso DW, Freeman S, Kuypers FA, Ambruso DR. A 29-kDa protein associated with p67phox expresses both peroxiredoxin and phospholipase A2 activity and enhances superoxide anion production by a cell-free system of NADPH oxidase activity. *J Biol Chem* 2002 November 22;277(47):45181-7.

(121) Lee JG, Lim EJ, Park DW, Lee SH, Kim JR, Baek SH. A combination of Lox-1 and Nox1 regulates TLR9-mediated foam cell formation. *Cell Signal* 2008 December;20(12):2266-75.

(122) Lee JI, Burckart GJ. Nuclear factor kappa B: important transcription factor and therapeutic target. *J Clin Pharmacol* 1998 November;38(11):981-93.

(123) Lee SH, Park DW, Park SC, Park YK, Hong SY, Kim JR, Lee CH, Baek SH. Calcium-independent phospholipase A2beta-Akt signaling is involved in lipopolysaccharide-induced NADPH oxidase 1 expression and foam cell formation. *J Immunol* 2009 December 1;183(11):7497-504.

(124) Lefer DJ, Scalia R, Jones SP, Sharp BR, Hoffmeyer MR, Farvid AR, Gibson MF, Lefer AM. HMG-CoA reductase inhibition protects the diabetic myocardium from ischemia-reperfusion injury. *FASEB J* 2001 June;15(8):1454-6.

(125) Lehel C, Olah Z, Petrovics G, Jakab G, Anderson WB. Influence of various domains of protein kinase C epsilon on its PMA-induced translocation from the Golgi to the plasma membrane. *Biochem Biophys Res Commun* 1996 June 5;223(1):98-103.

(126) Leonard EJ, Skeel A, Yoshimura T. Biological aspects of monocyte chemoattractant protein-1 (MCP-1). *Adv Exp Med Biol* 1991;305:57-64.

(127) Levonen AL, Vahakangas E, Koponen JK, Yla-Herttuala S. Antioxidant gene therapy for cardiovascular disease: current status and future perspectives. *Circulation* 2008 April 22;117(16):2142-50.

(128) Li N, McLaren JE, Michael DR, Clement M, Fielding CA, Ramji DP. ERK is integral to the IFN-gamma-mediated activation of STAT1, the expression of key genes implicated in atherosclerosis, and the uptake of modified lipoproteins by human macrophages. *J Immunol* 2010 September 1;185(5):3041-8.

(129) Liang CJ, Wang SH, Chen YH, Chang SS, Hwang TL, Leu YL, Tseng YC, Li CY, Chen YL. Viscolin reduces VCAM-1 expression in TNF-alpha-treated endothelial cells via the JNK/NF-kappaB and ROS pathway. *Free Radic Biol Med* 2011 October 1;51(7):1337-46.

(130) Liang P, MacRae TH. Molecular chaperones and the cytoskeleton. *J Cell Sci* 1997 July;110 (Pt 13):1431-40.

(131) Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002 March 5;105(9):1135-43.

(132) Lim JC, Choi HI, Park YS, Nam HW, Woo HA, Kwon KS, Kim YS, Rhee SG, Kim K, Chae HZ. Irreversible oxidation of the active-site cysteine of peroxiredoxin to cysteine sulfonic acid for enhanced molecular chaperone activity. *J Biol Chem* 2008 October 24;283(43):28873-80.

(133) Liu DH, Yuan HY, Cao CY, Gao ZP, Zhu BY, Huang HL, Liao DF. Heat shock protein 90 acts as a molecular chaperone in late-phase activation of extracellular signal-regulated kinase 1/2 stimulated by oxidative stress in vascular smooth muscle cells. *Acta Pharmacol Sin* 2007 December;28(12):1907-13.

(134) Liu W, Nakamura H, Shioji K, Tanito M, Oka S, Ahsan MK, Son A, Ishii Y, Kishimoto C, Yodoi J. Thioredoxin-1 ameliorates myosin-induced autoimmune myocarditis by suppressing chemokine expressions and leukocyte chemotaxis in mice. *Circulation* 2004 September 7;110(10):1276-83.

(135) Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* 2006 May 27;367(9524):1747-57.

(136) Lopez-Franco O, Hernandez-Vargas P, Ortiz-Munoz G, Sanjuan G, Suzuki Y, Ortega L, Blanco J, Egido J, Gomez-Guerrero C. Parthenolide modulates the NF-kappaB-mediated inflammatory responses in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006 August;26(8):1864-70.

(137) Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, Devera ME, Liang X, Tor M, Billiar T. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol Rev* 2007 December;220:60-81.

(138) Lu Y, Wahl LM. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. *J Immunol* 2005 October 15;175(8):5423-9.

(139) Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol* 2005 January;25(1):29-38.

(140) Malhotra V, Shanley TP, Pittet JF, Welch WJ, Wong HR. Geldanamycin inhibits NF-kappaB activation and interleukin-8 gene expression in cultured human respiratory epithelium. *Am J Respir Cell Mol Biol* 2001 July;25(1):92-7.

(141) Manea A, Raicu M, Simionescu M. Expression of functionally phagocyte-type NAD(P)H oxidase in pericytes: effect of angiotensin II and high glucose. *Biol Cell* 2005 September;97(9):723-34.

(142) Martin-Ventura JL, Blanco-Colio LM, Munoz-Garcia B, Gomez-Hernandez A, Arribas A, Ortega L, Tunon J, Egido J. NF-kappaB activation and Fas ligand overexpression in blood and plaques of patients with carotid atherosclerosis: potential implication in plaque instability. *Stroke* 2004 February;35(2):458-63.

(143) Martin-Ventura JL, Nicolas V, Houard X, Blanco-Colio LM, Leclercq A, Egido J, Vranckx R, Michel JB, Meilhac O. Biological significance of decreased HSP27 in human atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006 June;26(6):1337-43.

(144) Martin-Ventura JL, Ortego M, Esbrit P, Hernandez-Presa MA, Ortega L, Egido J. Possible role of parathyroid hormone-related protein as a proinflammatory cytokine in atherosclerosis. *Stroke* 2003 July;34(7):1783-9.

(145) Martinez-Pinna R, Lindholt JS, Blanco-Colio LM, Dejouvencel T, Madrigal-Matute J, Ramos-Mozo P, Vega de CM, Michel JB, Egido J, Meilhac O, Martin-Ventura JL. Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAAs). A potential link of oxidative stress with AAA evolution. *Atherosclerosis* 2010 June 1.

(146) Martinez-Pinna R, Ramos-Mozo P, Madrigal-Matute J, Blanco-Colio LM, Lopez JA, Calvo E, Camafeita E, Lindholt JS, Meilhac O, Delbosc S, Michel JB, de Ceniga MV, Egido J, Martin-Ventura JL. Identification of peroxiredoxin-1 as a novel biomarker of abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol* 2011 April;31(4):935-43.

(147) Mayr M, Chung YL, Mayr U, Yin X, Ly L, Troy H, Fredericks S, Hu Y, Griffiths JR, Xu Q. Proteomic and metabolomic analyses of atherosclerotic vessels from apolipoprotein E-deficient mice reveal alterations in inflammation, oxidative stress, and energy metabolism. *Arterioscler Thromb Vasc Biol* 2005 October;25(10):2135-42.

(148) Meister A, Anderson ME. Glutathione. *Annu Rev Biochem* 1983;52:711-60.

(149) Meng QR, Gideon KM, Harbo SJ, Renne RA, Lee MK, Brys AM, Jones R. Gene expression profiling in lung tissues from mice exposed to cigarette smoke, lipopolysaccharide, or smoke plus lipopolysaccharide by inhalation. *Inhal Toxicol* 2006 July;18(8):555-68.

(150) Michel JB, Martin-Ventura JL, Egido J, Sakalihasan N, Treska V, Lindholt J, Allaire E, Thorsteinsdottir U, Cockerill G, Swedenborg J. Novel aspects of the pathogenesis of aneurysms of the abdominal aorta in humans. *Cardiovasc Res* 2011 April 1;90(1):18-27.

(151) Michel JB, Virmani R, Arbustini E, Pasterkamp G. Intraplaque haemorrhages as the trigger of plaque vulnerability. *Eur Heart J* 2011 August;32(16):1977-85, 1985a, 1985b, 1985c.

(152) Minami Y, Hohfeld J, Ohtsuka K, Hartl FU. Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40. *J Biol Chem* 1996 August 9;271(32):19617-24.

(153) Mirabelli F, Salis A, Perotti M, Taddei F, Bellomo G, Orrenius S. Alterations of surface morphology caused by the metabolism of menadione in mammalian cells are associated with the oxidation of critical sulfhydryl groups in cytoskeletal proteins. *Biochem Pharmacol* 1988 September 15;37(18):3423-7.

(154) Miyamoto S, Sakamoto T, Soejima H, Shimomura H, Kajiwara I, Kojima S, Hokamaki J, Sugiyama S, Yoshimura M, Ozaki Y, Nakamura H, Yodoi J, Ogawa H. Plasma thioredoxin levels and platelet aggregability in patients with acute myocardial infarction. *Am Heart J* 2003 September;146(3):465-71.

(155) Mohan S, Konopinski R, Yan B, Centonze VE, Natarajan M. High glucose-induced IKK-Hsp-90 interaction contributes to endothelial dysfunction. *Am J Physiol Cell Physiol* 2009 January;296(1):C182-C192.

(156) Moon EJ, Sonveaux P, Porporato PE, Danhier P, Gallez B, Batinic-Haberle I, Nien YC, Schroeder T, Dewhirst MW. NADPH oxidase-mediated reactive oxygen species production activates hypoxia-inducible factor-1 (HIF-1) via the ERK pathway after hyperthermia treatment. *Proc Natl Acad Sci U S A* 2010 November 23;107(47):20477-82.

(157) Morimoto RI, Kline MP, Bimston DN, Cotto JJ. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays Biochem* 1997;32:17-29.

(158) Mounier N, Arrigo AP. Actin cytoskeleton and small heat shock proteins: how do they interact? *Cell Stress Chaperones* 2002 April;7(2):167-76.

(159) Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. *J Biol Chem* 2008 January 18;283(3):1622-7.

(160) Nakamura H, Herzenberg LA, Bai J, Araya S, Kondo N, Nishinaka Y, Herzenberg LA, Yodoi J. Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. *Proc Natl Acad Sci U S A* 2001 December 18;98(26):15143-8.

(161) Nakamura H, Matsuda M, Furuke K, Kitaoka Y, Iwata S, Toda K, Inamoto T, Yamaoka Y, Ozawa K, Yodoi J. Adult T cell leukemia-derived factor/human thioredoxin protects endothelial F-2 cell injury caused by activated neutrophils or hydrogen peroxide. *Immunol Lett* 1994 September;42(1-2):75-80.

(162) Nakata A, Nakagawa Y, Nishida M, Nozaki S, Miyagawa J, Nakagawa T, Tamura R, Matsumoto K, Kameda-Takemura K, Yamashita S, Matsuzawa Y. CD36, a novel receptor for oxidized low-density lipoproteins, is highly expressed on lipid-laden macrophages in human atherosclerotic aorta. *Arterioscler Thromb Vasc Biol* 1999 May;19(5):1333-9.

(163) Neidhardt FC, VanBogelen RA, Vaughn V. The genetics and regulation of heat-shock proteins. *Annu Rev Genet* 1984;18:295-329.

(164) Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. *Nature* 2003 July 31;424(6948):561-5.

(165) Ni W, Egashira K, Kitamoto S, Kataoka C, Koyanagi M, Inoue S, Imaizumi K, Akiyama C, Nishida KI, Takeshita A. New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 2001 April 24;103(16):2096-101.

(166) Nichols WW, Pepine CJ, O'Rourke MF. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke. *N Engl J Med* 1999 June 3;340(22):1762-3.

(167) Nishihira K, Yamashita A, Imamura T, Hatakeyama K, Sato Y, Nakamura H, Yodoi J, Ogawa H, Kitamura K, Asada Y. Thioredoxin in coronary culprit lesions: possible relationship to oxidative stress and intraplaque hemorrhage. *Atherosclerosis* 2008 December;201(2):360-7.

(168) Okada M, Hasebe N, Aizawa Y, Izawa K, Kawabe J, Kikuchi K. Thermal treatment attenuates neointimal thickening with enhanced expression of heat-shock protein 72 and suppression of oxidative stress. *Circulation* 2004 April 13;109(14):1763-8.

(169) Okuda M, Inoue N, Azumi H, Seno T, Sumi Y, Hirata K, Kawashima S, Hayashi Y, Itoh H, Yodoi J, Yokoyama M. Expression of glutaredoxin in human coronary arteries: its potential role in antioxidant protection against atherosclerosis. *Arterioscler Thromb Vasc Biol* 2001 September;21(9):1483-7.

(170) Ortiz-Munoz G, Martin-Ventura JL, Hernandez-Vargas P, Mallavia B, Lopez-Parra V, Lopez-Franco O, Munoz-Garcia B, Fernandez-Vizarra P, Ortega L, Egido J, Gomez-Guerrero C. Suppressors of cytokine signaling modulate JAK/STAT-mediated cell responses during atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009 April;29(4):525-31.

(171) Pacey S, Gore M, Chao D, Banerji U, Larkin J, Sarker S, Owen K, Asad Y, Raynaud F, Walton M, Judson I, Workman P, Eisen T. A Phase II trial of 17-allylamino, 17-demethoxygeldanamycin (17-AAG, tanespimycin) in patients with metastatic melanoma. *Invest New Drugs* 2010 August 5.

(172) Palinski W, Napoli C. The fetal origins of atherosclerosis: maternal hypercholesterolemia, and cholesterol-lowering or antioxidant treatment during pregnancy influence in utero programming and postnatal susceptibility to atherogenesis. *FASEB J* 2002 September;16(11):1348-60.

(173) Paravicini TM, Touyz RM. Redox signaling in hypertension. *Cardiovasc Res* 2006 July 15;71(2):247-58.

(174) Parcellier A, Brunet M, Schmitt E, Col E, Didelot C, Hammann A, Nakayama K, Nakayama KI, Khochbin S, Solary E, Garrido C. HSP27 favors ubiquitination and proteasomal degradation of p27Kip1 and helps S-phase re-entry in stressed cells. *FASEB J* 2006 June;20(8):1179-81.

(175) Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem Biophys Res Commun* 2003 May 9;304(3):505-12.

(176) Park YM, Febbraio M, Silverstein RL. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *J Clin Invest* 2009 January;119(1):136-45.

(177) Pekkari K, Avila-Carino J, Bengtsson A, Gurunath R, Scheynius A, Holmgren A. Truncated thioredoxin (Trx80) induces production of interleukin-12 and enhances CD14 expression in human monocytes. *Blood* 2001 May 15;97(10):3184-90.

(178) Pekkari K, Gurunath R, Arner ES, Holmgren A. Truncated thioredoxin is a mitogenic cytokine for resting human peripheral blood mononuclear cells and is present in human plasma. *J Biol Chem* 2000 December 1;275(48):37474-80.

(179) Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci U S A* 1992 May 15;89(10):4471-5.

(180) Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992 October 16;71(2):343-53.

(181) Polla BS, Kantengwa S, Francois D, Salvioli S, Franceschi C, Marsac C, Cossarizza A. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc Natl Acad Sci U S A* 1996 June 25;93(13):6458-63.

(182) Polverini PJ, Cotran PS, Gimbrone MA, Jr., Unanue ER. Activated macrophages induce vascular proliferation. *Nature* 1977 October 27;269(5631):804-6.

(183) Popowich DA, Vavra AK, Walsh CP, Bhikhapurwala HA, Rossi NB, Jiang Q, Aalami OO, Kibbe MR. Regulation of reactive oxygen species by p53: implications for nitric oxide-mediated apoptosis. *Am J Physiol Heart Circ Physiol* 2010 June;298(6):H2192-H2200.

(184) Poulaki V, Iliaki E, Mitsiades N, Mitsiades CS, Paulus YN, Bula DV, Gragoudas ES, Miller JW. Inhibition of Hsp90 attenuates inflammation in endotoxin-induced uveitis. *FASEB J* 2007 July;21(9):2113-23.

(185) Powers ET, Morimoto RI, Dillin A, Kelly JW, Balch WE. Biological and chemical approaches to diseases of proteostasis deficiency. *Annu Rev Biochem* 2009;78:959-91.

(186) Powers MV, Workman P. Inhibitors of the heat shock response: biology and pharmacology. *FEBS Lett* 2007 July 31;581(19):3758-69.

(187) Powis G, Briehl M, Oblong J. Redox signalling and the control of cell growth and death. *Pharmacol Ther* 1995;68(1):149-73.

(188) Powis G, Montfort WR. Properties and biological activities of thioredoxins. *Annu Rev Biophys Biomol Struct* 2001;30:421-55.

(189) Powis G, Oblong JE, Gasdaska PY, Berggren M, Hill SR, Kirkpatrick DL. The thioredoxin/thioredoxin reductase redox system and control of cell growth. *Oncol Res* 1994;6(10-11):539-44.

(190) Pratt WB, Morishima Y, Peng HM, Osawa Y. Proposal for a role of the Hsp90/Hsp70-based chaperone machinery in making triage decisions when proteins undergo oxidative and toxic damage. *Exp Biol Med (Maywood)* 2010 March;235(3):278-89.

(191) Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med (Maywood)* 2003 February;228(2):111-33.

(192) Preville X, Salvemini F, Giraud S, Chaufour S, Paul C, Stepien G, Ursini MV, Arrigo AP. Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. *Exp Cell Res* 1999 February 25;247(1):61-78.

(193) Qu A, Jiang C, Xu M, Zhang Y, Zhu Y, Xu Q, Zhang C, Wang X. PGC-1alpha attenuates neointimal formation via inhibition of vascular smooth muscle cell migration in the injured rat carotid artery. *Am J Physiol Cell Physiol* 2009 September;297(3):C645-C653.

(194) Rahaman SO, Lennon DJ, Febbraio M, Podrez EA, Hazen SL, Silverstein RL. A CD36-dependent signaling cascade is necessary for macrophage foam cell formation. *Cell Metab* 2006 September;4(3):211-21.

(195) Ramanathan RK, Egorin MJ, Erlichman C, Remick SC, Ramalingam SS, Naret C, Holleran JL, TenEyck CJ, Ivy SP, Belani CP. Phase I pharmacokinetic and pharmacodynamic study of 17-dimethylaminoethylamino-17-demethoxygeldanamycin,

an inhibitor of heat-shock protein 90, in patients with advanced solid tumors. *J Clin Oncol* 2010 March 20;28(9):1520-6.

(196) Ranford JC, Henderson B. Chaperonins in disease: mechanisms, models, and treatments. *Mol Pathol* 2002 August;55(4):209-13.

(197) Recinos A, III, LeJeune WS, Sun H, Lee CY, Tieu BC, Lu M, Hou T, Boldogh I, Tilton RG, Brasier AR. Angiotensin II induces IL-6 expression and the Jak-STAT3 pathway in aortic adventitia of LDL receptor-deficient mice. *Atherosclerosis* 2007 September;194(1):125-33.

(198) Redgrave JN, Gallagher P, Lovett JK, Rothwell PM. Critical cap thickness and rupture in symptomatic carotid plaques: the oxford plaque study. *Stroke* 2008 June;39(6):1722-9.

(199) Rhee SG. Redox signaling: hydrogen peroxide as intracellular messenger. *Exp Mol Med* 1999 June 30;31(2):53-9.

(200) Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 2005 June 15;38(12):1543-52.

(201) Rhee SG, Jeong W, Chang TS, Woo HA. Sulfiredoxin, the cysteine sulfinic acid reductase specific to 2-Cys peroxiredoxin: its discovery, mechanism of action, and biological significance. *Kidney Int Suppl* 2007 August;(106):S3-S8.

(202) Rhee SG, Kang SW, Jeong W, Chang TS, Yang KS, Woo HA. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Curr Opin Cell Biol* 2005 April;17(2):183-9.

(203) Rhee SG, Woo HA. Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger HO, and protein chaperones. *Antioxid Redox Signal* 2011 August 1;15(3):781-94.

(204) Rice JW, Veal JM, Fadden RP, Barabasz AF, Partridge JM, Barta TE, Dubois LG, Huang KH, Mabbett SR, Silinski MA, Steed PM, Hall SE. Small molecule inhibitors of Hsp90 potently affect inflammatory disease pathways and exhibit activity in models of rheumatoid arthritis. *Arthritis Rheum* 2008 December;58(12):3765-75.

(205) Ritossa F. Discovery of the heat shock response. *Cell Stress Chaperones* 1996 June;1(2):97-8.

(206) Rosenblat M, Aviram M. Macrophage glutathione content and glutathione peroxidase activity are inversely related to cell-mediated oxidation of LDL: in vitro and in vivo studies. *Free Radic Biol Med* 1998 January 15;24(2):305-17.

(207) Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999 January 14;340(2):115-26.

(208) Rubartelli A, Bajetto A, Allavena G, Wollman E, Sitia R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J Biol Chem* 1992 December 5;267(34):24161-4.

(209) Sahaf B, Rosen A. Secretion of 10-kDa and 12-kDa thioredoxin species from blood monocytes and transformed leukocytes. *Antioxid Redox Signal* 2000;2(4):717-26.

(210) Salminen A, Lehtonen M, Paimela T, Kaarniranta K. Celastrol: Molecular targets of Thunder God Vine. *Biochem Biophys Res Commun* 2010 April 9;394(3):439-42.

(211) Sarge KD, Murphy SP, Morimoto RI. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Mol Cell Biol* 1993 March;13(3):1392-407.

(212) Sato A, Hoshino Y, Hara T, Muro S, Nakamura H, Mishima M, Yodoi J. Thioredoxin-1 ameliorates cigarette smoke-induced lung inflammation and emphysema in mice. *J Pharmacol Exp Ther* 2008 May;325(2):380-8.

(213) Sato M, Fraga C, Das DK. Induction of the expression of cardioprotective proteins after mild-to-moderate consumption of alcohol. *Pathophysiology* 2004 April;10(2):139-45.

(214) Schuett H, Luchtefeld M, Grothusen C, Grote K, Schieffer B. How much is too much? Interleukin-6 and its signalling in atherosclerosis. *Thromb Haemost* 2009 August;102(2):215-22.

(215) Schulze PC, Yoshioka J, Takahashi T, He Z, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* 2004 July 16;279(29):30369-74.

(216) Schwartz SM, deBlois D, O'Brien ER. The intima. Soil for atherosclerosis and restenosis. *Circ Res* 1995 September;77(3):445-65.

(217) Schwartz SM, Virmani R, Rosenfeld ME. The good smooth muscle cells in atherosclerosis. *Curr Atheroscler Rep* 2000 September;2(5):422-9.

(218) Seki Y, Kai H, Shibata R, Nagata T, Yasukawa H, Yoshimura A, Imaizumi T. Role of the JAK/STAT pathway in rat carotid artery remodeling after vascular injury. *Circ Res* 2000 July 7;87(1):12-8.

(219) Sepp-Lorenzino L, Ma Z, Lebwohl DE, Vinitsky A, Rosen N. Herbimycin A induces the 20 S proteasome- and ubiquitin-dependent degradation of receptor tyrosine kinases. *J Biol Chem* 1995 July 14;270(28):16580-7.

(220) Shao LE, Tanaka T, Gribi R, Yu J. Thioredoxin-related regulation of NO/NOS activities. *Ann N Y Acad Sci* 2002 May;962:140-50.

(221) Shi Y, Mosser DD, Morimoto RI. Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev* 1998 March 1;12(5):654-66.

(222) Shimizu H, Hirose Y, Nishijima F, Tsubakihara Y, Miyazaki H. ROS and PDGF-beta [corrected] receptors are critically involved in indoxyl sulfate actions that promote vascular smooth muscle cell proliferation and migration. *Am J Physiol Cell Physiol* 2009 August;297(2):C389-C396.

(223) Shringarpure R, Grune T, Mehlhase J, Davies KJ. Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J Biol Chem* 2003 January 3;278(1):311-8.

(224) Shull S, Heintz NH, Periasamy M, Manohar M, Janssen YM, Marsh JP, Mossman BT. Differential regulation of antioxidant enzymes in response to oxidants. *J Biol Chem* 1991 December 25;266(36):24398-403.

(225) Slaby I, Holmgren A. Thioredoxin reductase-dependent insulin disulfide reduction by phage T7 DNA polymerase reflects dissociation of the enzyme into subunits. *J Biol Chem* 1989 October 5;264(28):16502-6.

(226) Smith DF, Sullivan WP, Marion TN, Zaitsu K, Madden B, McCormick DJ, Toft DO. Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. *Mol Cell Biol* 1993 February;13(2):869-76.

(227) Smith V, Sausville EA, Camalier RF, Fiebig HH, Burger AM. Comparison of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17DMAG) and 17-allylamino-17-demethoxygeldanamycin (17AAG) in vitro: effects on Hsp90 and client proteins in melanoma models. *Cancer Chemother Pharmacol* 2005 August;56(2):126-37.

(228) Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 2002 March 26;105(12):1429-35.

(229) Soti C, Nagy E, Giricz Z, Vigh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. *Br J Pharmacol* 2005 November;146(6):769-80.

(230) Spielberger JC, Moody AD, Watson WH. Oxidation and nuclear localization of thioredoxin-1 in sparse cell cultures. *J Cell Biochem* 2008 August 1;104(5):1879-89.

(231) Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W, Jr., Richardson M, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, . A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 1992 January;85(1):391-405.

(232) Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 1995 September 1;92(5):1355-74.

(233) Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Jr., Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb* 1994 May;14(5):840-56.

(234) Staubach S, Razawi H, Hanisch FG. Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7. *Proteomics* 2009 May;9(10):2820-35.

(235) Suematsu Y, Nishizawa Y, Shioi A, Hino M, Tahara H, Inaba M, Morii H, Otani S. Effect of 1,25-dihydroxyvitamin D3 on induction of scavenger receptor and differentiation of 12-O-tetradecanoylphorbol-13-acetate-treated THP-1 human monocyte like cells. *J Cell Physiol* 1995 December;165(3):547-55.

(236) Sun J, Liao JK. Induction of angiogenesis by heat shock protein 90 mediated by protein kinase Akt and endothelial nitric oxide synthase. *Arterioscler Thromb Vasc Biol* 2004 December;24(12):2238-44.

(237) Supko JG, Hickman RL, Grever MR, Malspeis L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother Pharmacol* 1995;36(4):305-15.

(238) Suzuki K, Tatsumi H, Satoh S, Senda T, Nakata T, Fujii J, Taniguchi N. Manganese-superoxide dismutase in endothelial cells: localization and mechanism of induction. *Am J Physiol* 1993 October;265(4 Pt 2):H1173-H1178.

(239) Takagi Y, Gon Y, Todaka T, Nozaki K, Nishiyama A, Sono H, Hashimoto N, Kikuchi H, Yodoi J. Expression of thioredoxin is enhanced in atherosclerotic plaques and during neointima formation in rat arteries. *Lab Invest* 1998 August;78(8):957-66.

(240) Takeya R, Ueno N, Kami K, Taura M, Kohjima M, Izaki T, Nunoi H, Sumimoto H. Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases. *J Biol Chem* 2003 July 4;278(27):25234-46.

(241) Tian ZQ, Liu Y, Zhang D, Wang Z, Dong SD, Carreras CW, Zhou Y, Rastelli G, Santi DV, Myles DC. Synthesis and biological activities of novel 17-aminogeldanamycin derivatives. *Bioorg Med Chem* 2004 October 15;12(20):5317-29.

(242) Tissieres A, Mitchell HK, Tracy UM. Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *J Mol Biol* 1974 April 15;84(3):389-98.

(243) Tissieres A, Mitchell HK, Tracy UM. Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *J Mol Biol* 1974 April 15;84(3):389-98.

(244) Trigona WL, Mullarky IK, Cao Y, Sordillo LM. Thioredoxin reductase regulates the induction of haem oxygenase-1 expression in aortic endothelial cells. *Biochem J* 2006 February 15;394(Pt 1):207-16.

(245) Umekawa T, Sugiyama T, Kihira T, Murabayashi N, Zhang L, Nagao K, Kamimoto Y, Ma N, Yodoi J, Sagawa N. Overexpression of thioredoxin-1 reduces oxidative stress in the placenta of transgenic mice and promotes fetal growth via glucose metabolism. *Endocrinology* 2008 August;149(8):3980-8.

(246) Ungvari Z, Csiszar A, Huang A, Kaminski PM, Wolin MS, Koller A. High pressure induces superoxide production in isolated arteries via protein kinase C-dependent activation of NAD(P)H oxidase. *Circulation* 2003 September 9;108(10):1253-8.

(247) Uwayama J, Hirayama A, Yanagawa T, Warabi E, Sugimoto R, Itoh K, Yamamoto M, Yoshida H, Koyama A, Ishii T. Tissue Prx I in the protection against Fe-NTA and the reduction of nitroxyl radicals. *Biochem Biophys Res Commun* 2006 January 6;339(1):226-31.

(248) Viles-Gonzalez JF, Fuster V, Badimon JJ. Atherothrombosis: a widespread disease with unpredictable and life-threatening consequences. *Eur Heart J* 2004 July;25(14):1197-207.

(249) Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2000 May;20(5):1262-75.

(250) von HP, Koenen RR, Sack M, Mause SF, Adriaens W, Proudfoot AE, Hackeng TM, Weber C. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood* 2005 February 1;105(3):924-30.

(251) Walsh D, Li Z, Wu Y, Nagata K. Heat shock and the role of the HSPs during neural plate induction in early mammalian CNS and brain development. *Cell Mol Life Sci* 1997 February;53(2):198-211.

(252) Wang Y, De Keulenaer GW, Lee RT. Vitamin D(3)-up-regulated protein-1 is a stress-responsive gene that regulates cardiomyocyte viability through interaction with thioredoxin. *J Biol Chem* 2002 July 19;277(29):26496-500.

(253) Wang Z, Chen Y, Labinskyy N, Hsieh TC, Ungvari Z, Wu JM. Regulation of proliferation and gene expression in cultured human aortic smooth muscle cells by resveratrol and standardized grape extracts. *Biochem Biophys Res Commun* 2006 July 21;346(1):367-76.

(254) Wax S, Piecyk M, Maritim B, Anderson P. Geldanamycin inhibits the production of inflammatory cytokines in activated macrophages by reducing the stability and translation of cytokine transcripts. *Arthritis Rheum* 2003 February;48(2):541-50.

(255) Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, Mason MD, Clayton A. Proteomics analysis of bladder cancer exosomes. *Mol Cell Proteomics* 2010 June;9(6):1324-38.

(256) Westermann P, Knoblich M, Maier O, Lindschau C, Haller H. Protein kinase C bound to the Golgi apparatus supports the formation of constitutive transport vesicles. *Biochem J* 1996 December 1;320 (Pt 2):651-8.

(257) Westwood JT, Wu C. Activation of Drosophila heat shock factor: conformational change associated with a monomer-to-trimer transition. *Mol Cell Biol* 1993 June;13(6):3481-6.

(258) Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer* 2005 October;5(10):761-72.

(259) Whitesell L, Mimnaugh EG, De CB, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci U S A* 1994 August 30;91(18):8324-8.

(260) Whitsett JA, Clark JC, Wispe JR, Pryhuber GS. Effects of TNF-alpha and phorbol ester on human surfactant protein and MnSOD gene transcription in vitro. *Am J Physiol* 1992 June;262(6 Pt 1):L688-L693.

(261) Wijayanti N, Naidu S, Kietzmann T, Immenschuh S. Inhibition of phorbol ester-dependent peroxiredoxin I gene activation by lipopolysaccharide via phosphorylation of RelA/p65 at serine 276 in monocytes. *Free Radic Biol Med* 2008 February 15;44(4):699-710.

(262) Wolfrum S, Grimm M, Heidbreder M, Dendorfer A, Katus HA, Liao JK, Richardt G. Acute reduction of myocardial infarct size by a hydroxymethyl glutaryl coenzyme A reductase inhibitor is mediated by endothelial nitric oxide synthase. *J Cardiovasc Pharmacol* 2003 March;41(3):474-80.

(263) Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 2003 April 25;300(5619):650-3.

(264) Wood ZA, Schroder E, Robin HJ, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 2003 January;28(1):32-40.

(265) World C, Spindel ON, Berk BC. Thioredoxin-interacting protein mediates TRX1 translocation to the plasma membrane in response to tumor necrosis factor-alpha: a key mechanism for vascular endothelial growth factor receptor-2 transactivation by reactive oxygen species. *Arterioscler Thromb Vasc Biol* 2011 August;31(8):1890-7.

(266) World CJ, Yamawaki H, Berk BC. Thioredoxin in the cardiovascular system. *J Mol Med* 2006 December;84(12):997-1003.

(267) Wormald S, Hilton DJ. Inhibitors of cytokine signal transduction. *J Biol Chem* 2004 January 9;279(2):821-4.

(268) Xu L, Giffard RG. HSP70 protects murine astrocytes from glucose deprivation injury. *Neurosci Lett* 1997 March 7;224(1):9-12.

(269) Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C, Neckers L. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc Natl Acad Sci U S A* 2002 October 1;99(20):12847-52.

(270) Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K, Rhee SG. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *J Biol Chem* 2002 October 11;277(41):38029-36.

(271) Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG. Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. *Ann N Y Acad Sci* 2005 August;1053:74-83.

(272) Yoshida S, Katoh T, Tetsuka T, Uno K, Matsui N, Okamoto T. Involvement of thioredoxin in rheumatoid arthritis: its costimulatory roles in the TNF-alpha-induced production of IL-6 and IL-8 from cultured synovial fibroblasts. *J Immunol* 1999 July 1;163(1):351-8.

(273) Yoshimura S, Morishita R, Hayashi K, Yamamoto K, Nakagami H, Kaneda Y, Sakai N, Ogihara T. Inhibition of intimal hyperplasia after balloon injury in rat carotid artery model using cis-element 'decoy' of nuclear factor-kappaB binding site as a novel molecular strategy. *Gene Ther* 2001 November;8(21):1635-42.

(274) Yoshimura T, Leonard EJ. Human monocyte chemoattractant protein-1: structure and function. *Cytokines* 1992;4:131-52.

(275) Zalba G, Beloqui O, San JG, Moreno MU, Fortuno A, Diez J. NADPH oxidase-dependent superoxide production is associated with carotid intima-media thickness in

subjects free of clinical atherosclerotic disease. *Arterioscler Thromb Vasc Biol* 2005 July;25(7):1452-7.

(276) Zalba G, Fortuno A, Orbe J, San JG, Moreno MU, Belzunce M, Rodriguez JA, Beloqui O, Paramo JA, Diez J. Phagocytic NADPH oxidase-dependent superoxide production stimulates matrix metalloproteinase-9: implications for human atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007 March;27(3):587-93.

(277) Zaragoza C, Gomez-Guerrero C, Martin-Ventura JL, Blanco-Colio L, Lavin B, Mallavia B, Tarin C, Mas S, Ortiz A, Egido J. Animal models of cardiovascular diseases. *J Biomed Biotechnol* 2011;2011:497841.

(278) Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med (Berl)* 2004 August;82(8):488-99.

(279) Zhang J, Li YD, Patel JM, Block ER. Thioredoxin overexpression prevents NO-induced reduction of NO synthase activity in lung endothelial cells. *Am J Physiol* 1998 August;275(2 Pt 1):L288-L293.

(280) Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992 October 16;258(5081):468-71.

(281) Zheng Z, Kim JY, Ma H, Lee JE, Yenari MA. Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. *J Cereb Blood Flow Metab* 2008 January;28(1):53-63.

(282) Zhu W, Roma P, Pellegatta F, Catapano AL. Oxidized-LDL induce the expression of heat shock protein 70 in human endothelial cells. *Biochem Biophys Res Commun* 1994 April 15;200(1):389-94.

(283) Zhu WM, Roma P, Pirillo A, Pellegatta F, Catapano AL. Oxidized LDL induce hsp70 expression in human smooth muscle cells. *FEBS Lett* 1995 September 18;372(1):1-5.

VII. ANEXO A

Los resultados presentados en esta tesis han sido publicados parcialmente en:

Artículos originales:

- **Madrigal-Matute J**, Fernandez-García CE, Gómez-Guerrero C, López-Franco O, Muñoz-García B, Ramos-Mozo P, Ortega L, Egido J, Blanco-Colio LM, Martín-Ventura JL. HSP90 inhibition by 17DMAG attenuates oxidative stress in experimental atherosclerosis. *Cardiovasc Res*. 2012, Apr 30; Epub ahead to print.

- **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-García B, Ramos-Mozo P, Ortega L, Egido J, Martín-Ventura JL. Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis. *Cardiovasc Res*. 2010 May 1;86(2):330-7.

- **Madrigal-Matute J**, Blanco-Colio LM, Fortuño A, Martinez-Pinna R, Ramos-Mozo P, Beloqui O, Egido J, Zalba G, Martin-Ventura JL. Peroxiredoxin-1/Thioredoxin levels are associated to intima media thickness and NADPH oxidase activity in subjects with subclinical atherosclerosis. *Arterioscler Thromb Vasc Biol*. Under revision, 2011.

Revisiones:

- **Madrigal-Matute J**, Martin-Ventura JL, Blanco-Colio LM, Egido J, Michel JB, Meilhac O. Heat-shock proteins in cardiovascular disease. *Adv Clin Chem*. 2011;54:1-43. Review.

- **Madrigal-Matute J**, Martinez-Pinna R, Fernandez-García CE, Ramos-Mozo P, Burillo-Ipiens E, Egido J, Blanco-Colio LM Martin-Ventura JL. Cell stress proteins in atherothrombosis. *Oxid Med Cell Longev, under revision.*

Otros trabajos realizados durante el desarrollo de la tesis:

- **Madrigal-Matute J**, Sandal Lindholt J, Fernandez-Garcia CE, Benito-Martin A, Elena Burillo E, Zalba Z, Beloqui O, Ortiz A, Egido J, Blanco-Colio LM, Martin-Ventura JL .Galectin-3, a biomarker linking oxidative stress and inflammation with the clinical outcomes of patients with atherothrombosis. *Arterioscler Thromb Vasc Biol, under revision.*

- Martin-Ventura, JL, **Madrigal-Matute J**, Martinez-Pinna R, Ramos-Mozo P, Blanco-Colio LM; Moreno JA, Tarin C, Burillo E, Fernandez-Garcia CE, Egido J, Meilhac O, Michel JB. Hemo-thrombus as a source of oxidative stress in human atherothrombosis: detoxifying mechanisms and potential therapeutic options. *Thromb Haemost, under revision.*

- Ramos-Mozo P, **Madrigal-Matute** J, Vega de Ceniga M, Blanco-Colio LM, Meilhac O, Feldman L, Michel JB, Clancy P, Golledge J, Norman PE, Egido J, Martin-Ventura JL. Increased plasma levels of NGAL, a marker of neutrophil activation, in patients with abdominal aortic aneurysm. *Atherosclerosis*. 2012 Feb;220(2):552-6.

- Ramos-Mozo P, **Madrigal-Matute** J, Martinez-Pinna R, Blanco-Colio LM, Antonio Lopez J, Camafeita E, Meilhac O, Michel JB, Aparicio C, de Ceniga MV, Egido J, Martín Ventura JL. Proteomic Analysis of Polymorphonuclear Neutrophils Identifies Catalase as a Novel Biomarker of Abdominal Aortic Aneurysm. *Arterioscler Thromb Vasc Biol.* 2011 Dec;31(12):3011-9.

- Martinez-Pinna R, Ramos-Mozo P, **Madrigal-Matute J**, Blanco-Colio LM, Lopez JA, Calvo E, Camafeita E, Lindholt JS, Meilhac O, Delbosc S, Michel JB, de Ceniga MV, Egido J, Martin-Ventura JL. Identification of peroxiredoxin-1 as a novel biomarker of abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol.* 2011 Apr;31(4):935-43.

- Muñoz-García B, **Madrigal-Matute J**, Moreno JA, Martin-Ventura JL, López-Franco O, Sastre C, Ortega L, Burkly LC, Egido J, Blanco-Colio LM. TWEAK-Fn14 interaction enhances plasminogen activator inhibitor 1 and tissue factor expression in atherosclerotic plaques and in cultured vascular smooth muscle cells. *Cardiovasc Res.* 2011 Jan 1;89(1):225-33.

- Martinez-Pinna R, Lindholt JS, Blanco-Colio LM, Dejouvencel T, **Madrigal-Matute J**, Ramos-Mozo P, Vega de Ceniga M, Michel JB, Egido J, Meilhac O, Martin-Ventura JL. Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAAs). A potential link of oxidative stress with AAA evolution. *Atherosclerosis*. 2010 Sep;212(1):333-8.

- Martín-Ventura JL, Blanco-Colio LM, Tuñón J, Muñoz-García B, **Madrigal-Matute J**, Moreno JA, Vega de Céniga M, Egido J. Biomarkers in cardiovascular medicine. *Rev Esp Cardiol.* 2009 Jun;62(6):677-88. Review.

- Moreno JA, Muñoz-García B, Martín-Ventura JL, **Madrigal-Matute J**, Orbe J, Páramo JA, Ortega L, Egido J, Blanco-Colio LM. The CD163-expressing macrophages recognize and internalize TWEAK: potential consequences in atherosclerosis. *Atherosclerosis*. 2009 Nov;207(1):103-10.

- Martín-Ventura JL, **Madrigal-Matute J**, Muñoz-Garcia B, Blanco-Colio LM, Van Oostrom M, Zalba G, Fortuño A, Gomez-Guerrero C, Ortega L, Ortiz A, Diez J, Egido J. Increased CD74 expression in human atherosclerotic plaques: contribution to inflammatory responses in vascular cells. *Cardiovasc Res*. 2009 Aug 1;83(3):586-94.

- Martín-Ventura JL, Muñoz-Garcia B, Blanco-Colio LM, Martín-Conejero A, **Madrigal-Matute J**, Vega M, Ortega L, Serrano J, Egido J. Treatment with amlodipine and atorvastatin has additive effect on blood and plaque inflammation in hypertensive patients with carotid atherosclerosis. *Kidney Int Suppl*. 2008 Dec;(111):S71-4.

Los resultados de esta tesis han sido parcialmente presentados en las siguientes reuniones científicas:

INTERNACIONALES

AUTORES/AS: **Madrigal-Matute J**, Blanco-Colio LM, Fortuño A, Martinez-Pinna R, Ramos-Mozo P, Beloqui O, Egido J, Zalba G, Martin-Ventura JL.
TÍTULO: *Peroxiredoxin-1/Thioredoxin levels are associated to intima media thickness and NADPH oxidase activity in subjects with subclinical atherosclerosis.*
TIPO DE PARTICIPACIÓN: ORAL
CONGRESO: **XVI International Symposium on Atherosclerosis 2012.**
PUBLICACIÓN: http://www.arinex.com.au/abstracts/isa2012/pdf/1203015Abstract00557.pdf
LUGAR DE CELEBRACIÓN: Sidney.
AÑO: 2012.

AUTORES/AS: **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-García B, Ramos-Mozo P, Ortega L, Egido J, Martín-Ventura JL.
TÍTULO: HSP90 inhibition by *17-DMAG reduces oxidative stress in experimental atheroslcerosis.*
TIPO DE PARTICIPACIÓN: ORAL

CONGRESO: **XVI International Symposium on Atherosclerosis 2012.**
PUBLICACIÓN: http://www.arinex.com.au/abstracts/isa2012/pdf/1203015Abstract00570.pdf
LUGAR DE CELEBRACIÓN: Sidney.
AÑO: 2012.

AUTORES/AS: **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-García B, Ramos-Mozo P, Ortega L, Egido J, Martín-Ventura JL.
TÍTULO: *17-DMAG reduces oxidative stress responses in atheroslcerosis.*
TIPO DE PARTICIPACIÓN: ORAL.
CONGRESO: **Fifth International Congress on Stress Responses in Biology and Medicine.**
PUBLICACIÓN: Fifth International Congress on Stress Responses in Biology and Medicine, lecturers´ abstract, SYMP-6-4, pp 78.
LUGAR DE CELEBRACIÓN: Quebec.
AÑO: 2011.

AUTORES/AS: **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-Garcia B, Ramos-Mozo P, Egido J, Martín-Ventura JL.
TÍTULO: *Heat shock protein (HSP)-90 inhibitors prevent the inflammatory response in atherogenesis.*
TIPO DE PARTICIPACIÓN: POSTER.
CONGRESO: **XV International Symposium on Atherosclerosis 2009.**
PUBLICACIÓN: XV International Symposium on Atherosclerosis 2009 Final program, Póster 678, pp 98.
LUGAR DE CELEBRACIÓN: Boston.
AÑO: 2009.

NACIONALES

AUTORES/AS: **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-Garcia B, Ramos-Mozo P, Gómez-Guerrero C, Egido J, Martín-Ventura JL.
TÍTULO: HSP90 inhibition by *17-DMAG reduces oxidative stress in experimental atheroslcerosis.*
TIPO DE PARTICIPACIÓN: PÓSTER.
CONGRESO: **XXV Congreso Nacional de la Sociedad Espanola de Arteriosclerosis (SEA).**
PUBLICACIÓN: -

LUGAR DE CELEBRACIÓN: Reus.
AÑO: 2012.

AUTORES/AS: **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-Garcia B, Ramos-Mozo P, Gómez-Guerrero C, Egido J, Martín-Ventura JL.
TÍTULO: *Peroxiredoxin-1/Thioredoxin levels are associated to intima media thickness and NADPH oxidase activity in subjects with subclinical atherosclerosis.*
TIPO DE PARTICIPACIÓN: PÓSTER.
CONGRESO: **XXIV Congreso Nacional de la Sociedad Espanola de Arteriosclerosis (SEA) y del XIII Congreso Internacional de la Sociedad Iberolatinoamericana de Arteriosclerosis (SILAT).**
PUBLICACIÓN: Clin Invest Arterioscl. 2011;23 (Espec Cong), pp. 59, Poster A-11.
LUGAR DE CELEBRACIÓN: Sevilla.
AÑO: 2011.

AUTORES/AS: **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-Garcia B, Ramos-Mozo P, Egido J, Martín-Ventura JL.
TÍTULO: Los inhibidores de la Heat shock protein (HSP)-90 previenen la respuesta inflamatoria durante aterogénesis.
TIPO DE PARTICIPACIÓN: PÓSTER.
CONGRESO: **XXII CONGRESO NACIONAL S.E.A.**
PUBLICACIÓN: Clin Invest Arterioscl. 2009; 21 (Espec Cong), pp. 40, Póster 16.
LUGAR DE CELEBRACIÓN: Pamplona.
AÑO: 2009.

AUTORES/AS: **Madrigal-Matute J**, López-Franco O, Blanco-Colio LM, Muñoz-Garcia B, Ramos-Mozo P, Egido J, Martín-Ventura JL.
TÍTULO: *LOS INHIBIDORES DE LA HEAT SHOCK PROTEIN (HSP)- 90 PREVIENEN LA RESPUESTA INFLAMATORIA DE CÉLULAS VASCULARES Y MONOCITOS HUMANOS.*
CONGRESO: **XXI CONGRESO NACIONAL S.E.A.**
PUBLICACIÓN: Clin Invest Arterioscl. 2008; 20, Extraordinario 3, pp. 40, Póster 108.
LUGAR DE CELEBRACIÓN, : Madrid.
AÑO: 2008.

VIII. ANEXO B

ADVANCES IN CLINICAL CHEMISTRY, VOL. 54

HEAT-SHOCK PROTEINS IN CARDIOVASCULAR DISEASE

Julio Madrigal-Matute,[*] Jose Luis Martin-Ventura,[*] Luis Miguel Blanco-Colio,[*] Jesus Egido,[*] Jean-Baptiste Michel,[†] and Olivier Meilhac[†,1]

[*]Vascular Research Lab, IIS, Fundación Jiménez Díaz, Autónoma University, Av. Reyes Católicos 2, Madrid, Spain
[†]Inserm U698, Hemostasis, Bio-engineering and Cardiovascular Remodeling, Hospital Bichat, Paris, France

Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-desmethoxy-17-*N*, *N*-dimethylaminoethylaminogeldanamycin
acLDL	acetylated LDL
ACS	acute coronary syndrome
AIF	apoptosis inducing factor

[1]Corresponding author: Olivier Meilhac, e-mail: olivier.meilhac@inserm.fr

0065-2423/11 $35.00
DOI: 10.1016/B978-0-12-387025-4.00001-7

AMI	acute myocardial infarction
Ang-II	angiotensin-II
APAF1	apoptosis protease activating factor 1
ApoE−/−	apolipoprotein E knock out
BAECs	bovine aortic endothelial cells
BMP	bone morphogenetic protein
CAD	coronary artery disease
CD	cluster of differentiation
CHD	coronary heart disease
CRP	C-reactive protein
CVD	cardiovascular disease
eEF2 kinase	eukaryotic elongation factor-2 kinase
eNOS	endothelial NOS
ERK	extracellular signal-regulated kinase
Foxp3	forkhead box P3
GSH	glutathione
HDF	human diploid fibroblasts
Hip	HSP70-interacting protein
HIV	human immunodeficiency virus
HO-1	heme oxygenase-1
Hop	HSP70–HSP90 organizing protein
HOPE study	the heart outcomes prevention evaluation study
HSE	heat-shock element
HSF	heat-shock factor
HSP	heat-shock protein
HSR	heat-shock response
HUVECs	human umbilical vein endothelial cells
Ig	immunoglobulin
IL	interleukin
kDA	kilodalton
LDL-R−/−	LDL receptor knock out
LDLs	low-density lipoproteins
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MCP1	monocyte chemoattractant protein 1
MGP	matrix Gla protein
MI	myocardial infarction
Mn-SOD	manganese superoxide dismutase
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	NO synthases

oxLDL	oxidized LDL
PAMPs	pathogen-associated molecular patterns
ROS	reactive oxygen species
SA	stable angina
SAPK/JNK	stress-activated protein kinase/c-Jun N-terminal kinase
siRNA	small interference RNA
SMCs	smooth muscle cells
SR	scavenger receptor
TGF-β	transfoming growth factor beta
Th2 cytokine	T-helper type 2 cytokine
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
Tregs	T regulatory cells
VEGF	vascular endothelial growth factor
VSMCs	vascular SMCs
YC1	3-(5′-hydroxymethyl-2′-furyl)-1-benzyl-indazol

1. Abstract

Heat-shock proteins (HSPs) belong to a group of highly conserved families of proteins expressed by all cells and organisms and their expression may be constitutive or inducible. They are generally considered as protective molecules against different types of stress and have numerous intracellular functions. Secretion or release of HSPs has also been described, and potential roles for extracellular HSPs reported. HSP expression is modulated by different stimuli involved in all steps of atherogenesis including oxidative stress, proteolytic aggression, or inflammation. Also, antibodies to HSPs may be used to monitor the response to different types of stress able to induce changes in HSP levels. In the present review, we will focus on the potential implication of HSPs in atherogenesis and discuss the limitations to the use of HSPs and anti-HSPs as biomarkers of atherothrombosis. HSPs could also be considered as potential therapeutic targets to reinforce vascular defenses and delay or avoid clinical complications associated with atherothrombosis.

2. Introduction

Heat-shock proteins (HSPs) belong to a group of highly conserved families of proteins expressed by all cells and organisms from bacteria to humans in response to a variety of different stress stimuli, including heavy metals, inflammatory cytokines, amino acid analogues, oxidative stress, or ischemia [1].

The name of "stress proteins" would be more appropriate than "HSPs" but for historical reasons, due to the discovery of genes of the HSP family in salivary gland cells of *Drosophila* subsequent to heat shock [2,3], this name is still in use today. HSP expression may be constitutive or inducible. HSPs are generally considered as protective molecules against different types of stress. They have numerous intracellular functions including roles as molecular chaperones, promoting correct protein folding of newly synthesized or denatured proteins [4], inhibitors of apoptosis [5], or maintainers of cellular integrity by stabilization of the cytoskeleton [6]. Secretion or release of HSPs has also been described, and potential roles for extracellular HSPs reported. The compartmentalization of HSPs and their role as markers or actors in atherosclerosis will be discussed in this chapter. Several other reviews deal with HSPs and cardiovascular disease [7] including cardiac protection [8] or neuroprotection [9]. In the present review, we will focus on the potential implication of HSPs in atherogenesis and atherothrombotic complications; we will discuss whether they may be considered as biomarkers, whether they participate in the etiology of vascular complications, as well as their potential use as therapeutic agents.

HSPs are classified according to their molecular weight, ranging from 10 to 110 kDa. However, a new nomenclature has been recently proposed [10]. The correspondences of the principal HSPs that we will discuss here are presented in Table 1, but the old nomenclature will be used throughout this review. Table 1 also summarizes the cardiovascular origin of the different HSPs, their potential inducers, their reported functions, and whether their circulating levels (both antigens and antibodies directed against HSPs) are associated with cardiovascular disease.

3. Atherogenesis and Possible Stimuli of Inducible HSPs

Several elements participating in atherogenesis have a strong impact on HSP expression and their posttranslational modifications, such as phosphorylation. We will summarize the different steps of atherogenesis leading to atherothrombotic complications and clinical manifestations with a particular emphasis on molecular events reported to induce HSP expression (Fig. 1).

The formation of atheroma starts during childhood by the accumulation of phagocytic cells in the intimal layer of the arterial wall. The intima is constituted by the endothelial layer and subjacent extracellular matrix, separated by the internal elastic lamina from the tunica media, principally composed of smooth muscle cells (SMCs), elastic, and collagen fibers in association with glycoproteins and proteoglycans. The intima represents a very limited space in healthy arteries where accumulation of phagocytic cells,

TABLE 1
HSPs: NEW NOMENCLATURE, CELL EXPRESSION, INDUCING FACTORS, INTRA/EXTRACELLULAR FUNCTIONS AND USE OF HSPs AS CIRCULATING BIOMARKERS

	New nomenclature	Cardiovascular expression	Induced by	Intracellular function	Extracellular function	Circulating biomarker	
						Antigen	Antibody
HSP60	HSPD [11]	Ubiquitously expressed [11]	Heat shock [12] miR-1/miR-206 [13] proinflammatory cytokines [12] Hemodynamic factors [14]	Cell survival [15] Apoptosis [16] Protein trafficking [17,18] Peptide hormone signaling [19] Proliferation [20]	Proinflammatory [21] Immunogen [22] Proapoptotic [16]	↑ in carotid atherosclerosis [23] Associated with IMT in borderline hypertension [24] Associated with severity of CAD [25,26] ↑ infection, stress, myocardial necrosis [27–29]	↑ carotid atherosclerosis [30] Associated with severity of CAD [25,31] ↓ MI compared to CHD [32] Predicitive of 5-year mortality in carotid atherosclerosis [30] ↑ higher risk of new CV event [33] Predicted coronary risk [34] Associated with infection [35] and CVD [36–40]
HSP70	HSPA [11]	Smooth muscle cells [41] Cardiac myocytes [42] Monocytes/ Macrophages [41]	Heat shock [12] Mechanical stress [43] Hyperlipidemia [44] oxLDL [45] HSP90 inhibitors [41] Other pharmacological compounds [46]	Antiinflammatory [41] Antiapoptotic [47] Antioxidant [48] Antiproliferative[46]	Proinflammatory [49] Proliferation and calcification [50] Immunogen [51]	↑ levels associated with decreased IMT in hypertensive patients [52] ↑ levels associated with low CAD risk [53] ↓ carotid atherosclerosis [54] Inversely correlated with neutrophil activation [54] ↑ ACS [55]	↓ CAD [56] not related with prevalence of CAD [53] and high risk of ACS [30]

(*continues*)

TABLE 1 (*Continued*)

	New nomenclature	Cardiovascular expression	Induced by	Intracellular function	Extracellular function	Circulating biomarker	
						Antigen	Antibody
HSP27	HSPB1 [11]	Smooth muscle cells [57] Endothelial cells [58] Cardiac myocytes [59,60] Monocytes/ Macrophages [61] Neutrophils [62]	Chemical stressors [58] Heat shock [63] Hyperlipidemia [44]	Actin stabilization [64] Muscle contraction [64] Cell migration [64] Cell survival [64] Antioxidant [65] Antiinflammatory [66]	Anti-inflammatory [61] Antiapoptotic [67] Antioxidant [62] Proapoptotic [62]	↓ atherosclerosis [68] ↑ ACS [69]	↑ MI in patients with ACS relative to unstable angina [70] ↑ acute chest pain [71]
HSP90	HSPC [11]	Monocytes/ macrophages [41] Smooth muscle cells [41] Endothelial cells [72]	Heat shock [73] Hyperlipidemia [44]	Antioxidant [74] Antiapoptotic [75] Pro-angiogenic [76] Proinflammatory [77]	Prooxidant [78] Proinflammatory [79]	↑ atherosclerosis [80]	↑ atherosclerosis [81]

↑ Increased
↓ Decreased

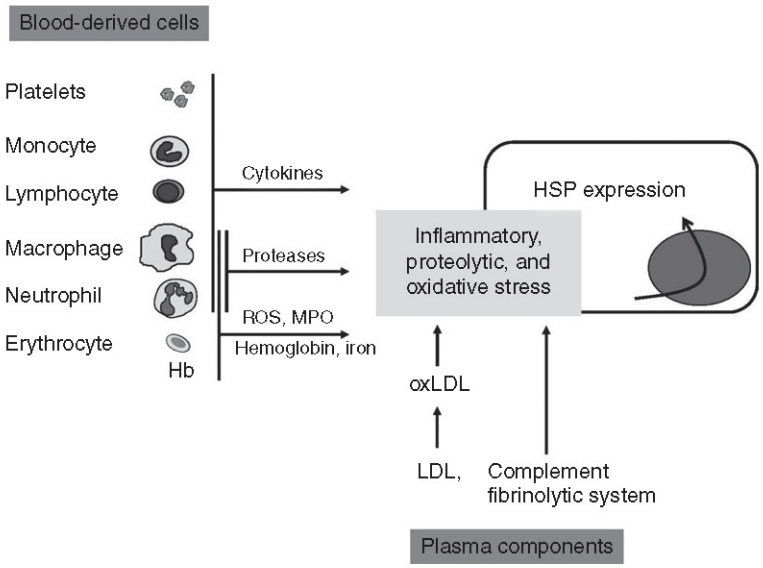


FIG. 1. HSP expression may be modulated by different types of stresses linked to atherogenesis [82] such as proteolytic aggression (e.g., HSP27 expression is increased in response to plasmin in human vascular smooth muscle cells, [57]), stimulation by cytokines [83], or oxidative stress. In particular, oxidized LDLs are reported to induce HSP expression [84]. Also, erythrophagocytosis was shown to induce the synthesis of different HSPs in human monocytes/macrophages [85]. Injection of lysed blood was reported to induce HSP70 expression in the brain [86], suggesting that free hemoglobin is able to trigger HSP expression. In response, HSPs may protect vascular cells against different types of aggression within the atherothrombotic plaque.

named foam cells due to their vacuolated aspect, has been detected in human fetal arteries, in particular in cases of maternal hypercholesterolemia [87]. Hypercholesterolemia is the major risk factor for the development of atheromatous disease. In particular, high circulating levels of low-density lipoproteins (LDLs) lead to their intimal deposition and the subsequent formation of foam cells due to nonregulated uptake of modified LDLs. The accumulation of foam cells produces fatty streaks observable in "en face" preparations of arterial samples. In humans, mutations of the LDL receptor (familial hypercholesterolemia) lead to a strong increase in plasma LDL concentration, thus favoring the development of atheromatous plaques and associated complications around the 3rd decade of life (myocardial infarction, stroke, etc.). Animal models deficient or mutated for the LDL receptor are commonly used as models of atherosclerosis (LDL-R knock-out mice, Watanabe heritable hyperlipidemic rabbits). LDLs, and in particular modified LDLs, have been reported to participate in all steps of atherogenesis. The major

modification of LDL shown to display atherogenic effects is their oxidation. LDLs and oxidized LDLs, as well as oxidative stress in general, are known to induce HSP expression in different cell types present in the pathological arterial wall (Fig. 1), which may constitute a response to injury.

Evolution of the fatty streaks toward fibroatheroma involves proliferation of SMCs within the intima that form the fibrous cap surrounding the foam cells and accumulated extracellular lipids (lipid core), characterized by a switch of the SMCs from a contractile to a secretory phenotype. HSPs could play a role in this step by interacting with the cytoskeletal proteins, such as actin, and thereby modifying SMC migration/proliferation [88]. Fibroatheromatous plaques evolve toward more complicated lesions that are very heterogeneous but often characterized by the presence of sclerotic material (calcifications) and the formation of a necrotic, lipidic, and hemocruoric core composed of cell debris, inflammatory, and blood cells (leukocytes, platelets, and red blood cells). HSPs may participate in processes associated with the evolution toward complicated plaques, such as calcification [50,89]. The presence of blood within the plaque was recently reported to be the major determinant of the clinical outcome in patients with carotid artery disease [90]. It reflects local plaque hemorrhage and is associated with increased intraplaque neovessels. Blood brings into the plaque both oxidative and proteolytic activities, which are the main driving forces of plaque vulnerability toward rupture, via fragilization of the fibrous cap and by inducing apoptosis of different vascular cells including SMCs. Many HSPs are induced in response to oxidative stress and proteolytic injury (Fig. 1); they may therefore constitute sensitive markers of these processes but also a response for restraining noxious insults potentially favoring plaque rupture and leading to clinical complications. These points will be discussed in detail in the present review.

4. HSPs/Anti-HSPs as Biomarkers of Atherothrombosis

Our definition of a biomarker is a marker reflecting or integrating one or several biological activities. Such markers may be any detectable and quantifiable molecules including proteins, peptides, lipids, nucleic acids, etc. This notion is of major importance when considering HSPs as potential biomarkers of cardiovascular disease. Biomarkers are not specific of a disease but rather reflect a biological activity associated with this pathology, at one time point. We will discuss the studies reporting differences in HSP expression in patients with atherosclerosis versus healthy subjects, directly by antigenic methods such as ELISA or Western blots in plasma or tissues, or indirectly by assessment of circulating antibodies raised against HSPs.

4.1. Antigenic Detection

4.1.1. *HSP60*

Different studies have analyzed the levels of circulating HSPs. Among them, levels of HSP60 are increased in patients with carotid atherosclerosis, suggesting its potential role as a diagnostic biomarker [23]. In patients with borderline hypertension, serum HSP60 levels were associated with intima–media thickness, a surrogate marker of atherosclerosis [24].

In addition, prospective data have confirmed an association between high levels of sHSP60 and early carotid atherosclerosis [91]. Similarly, another study has undertaken a prospective analysis of the association of HSP60 with the severity of CAD, reporting that HSP60 levels were significantly correlated with both the extent index and stenoses [25]. These data have been recently confirmed in a large case–control study, suggesting that the combination of HSP60 and anti-HSP60 antibody levels may predict this risk [26]. Potential explanations for the high HSP60 levels observed in atherosclerotic patients may be responses to infection, stress, or myocardial necrosis [27–29]. In complement to the clinical observation of increased HSP antigens in patients with atherothrombosis, different authors have analyzed the presence of HSPs in atherosclerotic plaques. In initial studies, increased HSP65 expression and the presence of HSP65-specific T-cells both in experimental and human atherosclerotic lesions were reported [14,92,93]. In subsequent studies, chlamydial HSP60 was colocalized with human HSP60 in plaque macrophages in human atherosclerotic lesions [94].

4.1.2. *HSP70*

An inverse relation between HSP70 and atherosclerosis has been reported by several groups. Whereas HSP70 is detectable in serum of nondiseased individuals [95], low serum HSP70 levels have been suggested to predict the development of atherosclerosis [52–54]. In hypertensive patients, increased concentrations of circulating HSP70 correlated with decreased intima/media thickness [52]. In another study by Zhu *et al.*, high serum levels of HSP70 were found to be associated with a low risk of coronary artery disease [53]. We have reported that plasma HSP70 concentrations were decreased in patients with carotid atherosclerosis relative to control healthy subjects [54]. Interestingly, circulating levels of neutrophil activation markers (myeloperoxidase, matrix metalloprotease 9/lipocalin complexes, and elastase) were inversely correlated with those of HSP70, suggesting the proteolytic degradation of this HSP under atherothrombotic conditions.

Under acute conditions, Zhang *et al.* recently reported that HSP70 was increased in patients with acute coronary syndrome (ACS) relative to age- and sex-matched healthy controls [55]. HSP70 levels were associated with

increased risk and severity of ACS. Interestingly, these authors monitored HSP70 levels at the time of admission, 2, 3, and 7 days after acute myocardial infarction (AMI). They report that HSP70 plasma concentration decreased rapidly after the onset of AMI. It is likely that following ischemia, the myocardial necrotic area releases large amounts of HSP70, as described in response to heat shock where HSP70 was abundant in small blood vessels found between the ventricular cardiomyocytes [96].

Berberian *et al.* first reported HSP70 expression in normal human aortas and carotid atherosclerotic plaques [97]. In atherosclerotic tissue, the necrotic core and its underlying media contained significantly more HSP70 staining than did fibrotic areas [47]. Accumulation of HSP70 in VSMCs adjacent to the necrotic core was suggested to be insufficient to protect them against the noxious stimuli of the plaque. We have recently quantified HSP70 immunostaining in 60 human atherosclerotic plaques and showed an increased expression of HSP70 in the shoulder region of the plaque compared to the fibrous area, probably reflecting increased stress of this vulnerable region due to blood flow. Interestingly, when atherosclerotic plaques were classified according to the cap thickness, we observed that HSP70 expression is lower in plaques with thin caps ($< 165\ \mu$m), suggesting that HSP70 plays an important role in the stability of advanced human atherosclerotic plaques [41].

4.1.3. *HSP27*

Plasma levels of HSP27 were shown to be decreased in atherosclerosis following a proteomic comparison between conditioned medium obtained from human carotid samples and healthy mammary endarteries [68]. At this time, HSP27 was described as an intracellular protein ubiquitously expressed by many cell types, including vascular cells. A noncandidate proteomic-based approach allowed us to discover HSP27 as a potential marker of nondiseased vascular wall. The decreased solubilization of HSP27 under atherothrombotic conditions was attributed, at least in part, to proteolytic activities such as that of plasmin present in culprit plaques and able to digest the soluble HSP27, potentially reducing its circulating levels [57].

In a prospective study including 255 female health care professionals devoid of cardiovascular disease at the time of plasma sampling, we were unable to show any association between baseline HSP27 plasma level and incidence of cardiovascular events (myocardial infarction, ischemic stroke, or cardiovascular death) during a follow-up period of up to 5.9 years [98]. These results may be explained by the apparently healthy state of the subjects at study initiation. Therefore, the results may not be applicable to other populations, such as those with advanced atherosclerosis or ACS.

Following a global proteomic approach on homogenized carotid samples, Park *et al.* [99] have also identified HSP27 as a protein which is overexpressed in the nearby normal-appearing area compared with the plaque core area. These authors showed that HSP27 plasma levels were increased in 27 patients with ACS relative to patients with stable angina (SA), patients with coronary risk factors, or healthy subjects. They concluded that increased HSP27 plasma levels may reflect the presence of vulnerable plaques. However, since blood was sampled within 24 h of the onset of ACS, it cannot be ruled out that the increase in HSP27 levels is secondary to myocardial ischemia or necrosis, as previously suggested for HSP70 [69].

By immunohistochemistry, we found that both human atherosclerotic plaques and mammary arteries expressed HSP27 protein [68]. Interestingly, HSP27 expression, which was mainly present in the cap and media colocalizing with alpha-actin-positive VSMCs, was inversely correlated with markers of apoptosis [57].

4.1.4. *HSP90*

In a recent paper, Businaro *et al.* have shown increased HSP90 serum levels in patients with atherosclerosis. HSP90 was overexpressed in plaques from patients with atherosclerosis, potentially contributing to plaque instability by inducing an immune response [81]. In agreement, we have shown an increased expression of HSP90 in the vulnerable region of human atherosclerotic plaques. Moreover, atherosclerotic plaques with thin caps ($<165\ \mu$m) displayed higher total HSP90 levels, suggesting that HSP90 correlates with events leading to the instability of advanced human atherosclerotic plaques [41].

As mentioned above, extensive research has been undertaken on circulating HSPs, reported to be either positively (HSP60) or negatively (HSP70) correlated with the presence and progression of atherosclerosis. HSP27 has been known for a long time for its antiapoptotic, antioxidant and thus antiatherogenic functions at a cellular level (discussed in more detail in the Section 4). However, further studies are needed to clarify the potential role of circulating HSP27 as a cardiovascular biomarker. More recently, HSP90 has also been associated with increased atherosclerosis. Only a few studies have addressed the predictive value of circulating HSPs in large patient cohorts. There is thus a need for such studies in the future. In relation to HSP expression in atherosclerotic plaques, it seems that whereas HSP70 and HSP27 are associated with features of plaque stability, HSP60 and HSP90 display the opposite pattern.

4.2. Indirect Detection via Anti-HSP Antibodies

Whereas HSP levels in plasma or serum may reflect transient variations in their secretion or release, detection of antibodies directed against HSPs could represent a more stable marker of a pathological state. Since HSPs are basically intracellular proteins, their presence in the extracellular compartment may trigger an immune response and lead to the production of anti-HSP antibodies. HSPs are highly conserved proteins that are also good immunogens.

4.2.1. *Anti-HSP60/65 Antibodies*

HSP65 is one of the most highly conserved proteins: 97% homology among prokaryotes and more than 70% homology between prokaryotic and human HSP65 [100]. Heat-shock proteins can promote, as well as regulate, autoimmunity. Therefore, antimicrobial HSP65 antibodies may cross-react with self-HSP65 [101]. It is thus difficult to clearly establish which antigen was originally responsible for the production of anti-HSP60/65 antibodies (microbial or self-source).

Several studies have suggested an association between antibodies directed against HSP60/65 (anti-HSP60/65) and atherothrombosis. In their earliest study, Xu *et al.* reported increased levels of serum antibodies against HSP65 in patients with carotid atherosclerosis [30]. In a subsequent study from the same group, HSP65 antibody titres were also increased in plasma of CAD patients whereas no correlation to established cardiovascular risk factors was observed. In contrast, HSP65 antibody levels were found to be significantly lower in AMI, compared to coronary heart disease (CHD) [32]. Following this study, Zhu *et al.* observed that anti-human HSP60 was also associated with the presence and severity of CAD [31]. In a recent study, anti-HSP60 was independently associated with CAD risk, and a combination of high anti-HSP60, hypertension, and diabetes was shown to be particularly detrimental for CAD risk [102]. The first study testing the potential prognostic value of HSP antibody levels showed that HSP65 antibody levels were predictive of 5-year mortality in patients with carotid atherosclerosis [30].

This initial observation was later confirmed in the HOPE study. Among patients with previous CV events or at high risk of such events, high serum concentration of antibodies to HSP65 was linked to a higher risk of developing new CV events during a mean follow-up of 4.5 years. This risk was even higher when combined with high levels of fibrinogen [33].

In another study, the authors observed that high IgA-class anti-HSP60 antibody levels predicted coronary risk, although the effect was modest without simultaneous occurrence of other classical risk factors [34].

Among potential explanations for the increased levels of antibodies to HSPs observed in plasma, infections might play an important role. Mayr *et al.* observed that anti-HSP65 antibody titres correlated with human IgA to *Chlamydia pneumoniae* and with IgG to *Helicobacter Pylori* [35]. In subsequent studies, high levels of antibodies to human HSP60 and *C. pneumoniae* were observed in coronary atherosclerosis, showing that their simultaneous presence substantially increased the risk for disease development [36]. Further, Heltai *et al.* demonstrated associations of high levels of anti-hHSP60 and anti-*C. pneumoniae* antibodies with AMI and of the level of anti-HSP65 antibodies with SA [37].

In addition, serum levels of anti-human HSP60 IgG antibody and anti-chlamydial IgM antibody, but not IgG or IgA, were significantly higher in ACS patients than in stable ischemic heart disease patients or controls [38]. Finally, antibodies to mycobacterial HSP65 are associated with elevated levels of coronary calcification and also correlated with *H. pylori* infection [39].

In relation to the potential prognostic value of HSP60 antibodies commented above, it was observed that a high level of HSP60 IgA could be considered as a risk factor for coronary events, especially when it was present together with *C. pneumoniae* infection and inflammation [40].

4.2.2. *Anti-HSP70 Antibodies*

In accordance with studies suggesting that increased levels of circulating HSP70 are correlated with a low risk of coronary artery disease, a publication by Hertz *et al.* reports that levels of antibodies directed against HSP70 are decreased in patients with CAD (SA and unstable angina) versus control subjects [56]. In contrast, a previous study by Zhu *et al.* did not find any association between anti-HSP70 IgG seropositivity and the prevalence of CAD despite decreased serum HSP70 levels in these patients [53]. More recently, Zhang *et al.* [30] reported that lower anti-HSP70 antibody levels are independently associated with a higher risk of ACS. To date, the association between anti-HSP70 levels and coronary artery disease is still unsettled and deserves further investigation.

4.2.3. *Anti-HSP27 Antibodies*

Antibody titres to HSP27 were reported to be elevated during the first 12 h following myocardial infarction in patients with ACS relative to patients with unstable angina [70]. These authors observed that anti-HSP27 antibody concentrations rapidly decrease during the 12–24 h period following MI. Shams *et al.* also reported increased anti-HSP27 titers in acute conditions, when patients where admitted to hospital with acute chest pain, as compared to patients without any history of CVD [71].

4.2.4. *Anti-HSP90 Antibodies*

Businaro *et al.* have recently shown increased HSP90 antibodies in serum from patients with atherosclerosis, implicating HSP90 as a possible autoantigen in the pathogenesis of carotid atherosclerosis [81].

4.2.5. *Limitations to the Use of HSPs and Anti-HSPs as Biomarkers of Atherothrombosis*

Since expression of inducible HSPs is dependent on a variety of stimuli, their levels may be modulated in different pathological states and even in physiological circumstances such as physical exercise [103,104]. For example, anti-HSP70 antibodies are increased in asthma [105], during HIV infection [106] or in patients with type II diabetes [107]. Also, increased titers of anti-HSP27 antibodies have been reported in women with ovarian cancer [108].

Detection of antibodies to bacterial HSPs, such as mHSP65, is not specific of an atherothrombotic state but rather reflects the presence of bacteria that may be independent of CVD. Although the implication of bacteria in atherogenesis has been suggested, further studies are needed to establish a causal link between infection and atherosclerosis [109]. Similarly, circulating HSPs may reflect a secretion by virtually all cell types. In spite of the direct access of arterial wall cells to the blood compartment, the release (or lack of release) of HSPs from focal atherothrombotic lesions may not have sufficient impact on their plasma concentrations to explain the differences observed between patients and subjects free of CVD. Therefore, plasma concentrations of HSPs may, as is the case for C-reactive protein (CRP), reflect a general state of stress or inflammation, not directly linked to atherothrombotic plaque evolution or vulnerability.

For example, the source of circulating HSP27 is still under debate since some authors could not detect it in cultured VSMCs whereas it is expressed by the medial layer in human artery samples [110]. However, incubation of human arteries devoid of atherosclerosis leads to a release of HSP27 in the conditioned medium, without trace of necrosis [68]. Macrophages can also be a source of HSP27; *in vitro*, human macrophages stimulated by estrogen secreted HSP27 via the exosomal pathway [111].

Since most HSPs are inducible, their expression and secretion may be rapidly modulated by an acute event. The above-mentioned work of Zhang *et al.* is a good example of the transient expression of HSPs [55]. These authors reported that plasma levels of HSP70 may predict risk of ACS which appears contradictory with all studies showing an inverse relation between high circulating levels of HSP70 and increased risk of atherothrombosis. Interpretation of the results should therefore take into account the time of blood sampling. It is likely that the expression of most HSPs,

including HSP70 and HSP27, is stimulated under acute conditions such as myocardial infarction.

HSP27 is a protein particularly easily detectable and identifiable by proteomic approaches. This protein is reported to be differentially expressed in many pathological situations. In fact, differential proteomics allowed identification of HSP27 as a potential marker of neuroblastoma [112], lymph node metastasis [113], chemotherapy response in patients with esophageal adenocarcinoma [114]. More than 120 publications are retrieved by a PubMed search when proteomics is combined with HSP27. Many pathological situations may module HSP expression and secretion. Caution must therefore be exercised before using HSPs as diagnostic or prognostic markers of any given disease.

5. Molecular Mechanisms: Bystanders or Actors?

In addition to their well-described chaperoning and antiapoptotic functions, HSPs play different roles depending upon their cellular location. The hypothesis of the Heat-Shock Paradox [1] is based on the idea that extracellular and intracellular HSPs exert different functions. While intracellular HSPs have been reported to downregulate inflammation [115–119], extracellular HSPs have been suggested, for the most part, to be proinflammatory by triggering an immune response [120,121]. This hypothesis may not apply to all HSPs as in the case of HSP27; its atheroprotective role has been shown in both intracellular and extracellular compartments [111]. In physiological conditions, HSPs play their main role of molecular chaperones promoting the correct folding of proteins. In pathological conditions, increased HSP levels may represent a response to modulate inflammation.

5.1. Intracellular Effects

Heat-shock response (HSR) is triggered by a variety of stress conditions that interfere with correct protein folding, leading to accumulation of misfolded or aggregated proteins. HSR is mediated by HSF1, a transcription factor which binds to heat-shock elements (HSE), present in the promoter region of a wide range of target genes, including HSPs [122]. Under normal conditions, HSP70 [80] and HSP90 [123] remain bound to monomeric HSF1 and some other cochaperones (i.e., HSP70–HSP90 Organizing Protein, Hop [124]; HSP70-Interacting Protein, Hip [125]; members of the HSP40/DnaJ HSPs family [126] or p23 [127]) in the cytoplasmic compartment. Under stress conditions, HSF1 is released, translocates to the nucleus, trimerizes and activates the synthesis of HSPs [128–130]. In fact, a negative feedback

mechanism modulates the stress response, since augmented levels of HSPs are able to sequester the free cytosolic HSF1 and therefore impede its translocation to the nucleus and the subsequent HSP synthesis. The complex interactions between the chaperones, cochaperones, and their client proteins decide the fate of a misfolded protein: either a new folding attempt or ubiquitination and subsequent degradation toward the proteasome pathway. However, in extreme oxidative conditions, ubiquitination can be bypassed [131].

Oxidative stress, inflammation, and apoptosis, among other processes, are involved in the initiation, development, and rupture of atherosclerotic plaques. Implication of HSPs in such events is gaining attention, and a number of studies are coming to light.

5.1.1. *HSP60*

Contradictory findings about the relationship of HSP60 with oxidative stress have been reported, although papers on this subject are scarce. Lee *et al.* raised this issue using normal human diploid fibroblasts (HDF) and found that sensitiveness to oxidative stress observed in young HDF cells was dependent upon HSP60 translocation from the mitochondria to the cytosol and subsequent massive activation of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) occurred [132]. In contrast, the use of a specific siRNA for HSP60 augmented resistance to oxidative stress [133].

As endothelial cells are the primary barrier in atherogenesis, HSP60 levels in endothelial cells were analyzed under stress conditions. HSP60 was expressed in the cytoplasm and on the surface of endothelial cells stressed by high temperature or TNF-α, and these cells were susceptible to complement-dependent lysis by HSP60-specific antibody [134]. Due to its association with infection, several studies analyzed the potential contribution of HSP60 in relation to bacteria/viruses in atherogenesis. Among them, *C. pneumoniae* was able to induce VSMC proliferation via HSP60 [20]. Also, during cytomegalovirus infection, antibodies against the virus can be generated, potentially cross-reacting with human HSP60 and leading to apoptosis of nonstressed endothelial cells [135]. Taking all these data into account, although the exact intracellular function of HSP60 is not clear, it may be considered as a potential mediator of oxidative stress and inflammation.

5.1.2. *HSP70*

5.1.2.1. *In vitro.* HSP70 has been suggested to exert antioxidative effects in cells exposed to H_2O_2 at the mitochondrial level [136], protecting cells by preserving levels of glutathione (GSH) [137]. Since endothelial damage after exposure to oxygen free radicals is considered to be important in the first steps of atherogenesis, HSP70 upregulation could be protective in these early stages of atherosclerosis. H_2O_2-induced oxidative stress in HUVECs was

significantly decreased after mild heat shock, which increased HSP70 mRNA and protein levels, providing delayed protection (up to 20 h) after preconditioning [48]. The protective role of HSP70 against inflammation has been previously reviewed [138]. Indeed, HSP70 involvement in the protection against inflammation in endothelial cells has been suggested [139], and we recently showed that HSP70 upregulation decreased inflammatory markers in macrophages and in VSMCs [41]. In addition, it was shown that an inducer of HSP70 (YC1: 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl-indazol) could effectively prevent VSMC proliferation induced by oxLDL [46].

The relation between HSP70 and apoptosis has been described in detail. APAF1 (apoptosis protease activating factor 1) binds to HSP70 and HSP90, thereby inhibiting the apoptotic signaling pathway [140–143]. HSP70 also inhibits AIF (apoptosis inducing factor) release from mitochondria [144,145]. In relation with CVD, inhibition of HSP70 expression has been shown to stimulate apoptosis and intimal hyperplasia in vein segments *ex vivo* by upregulating manganese superoxide dismutase (Mn-SOD) activity, an enzyme that protects mitochondria from injury in myocardial ischemia–reperfusion [146]. *In vitro* experiments performed by Wang *et al.* demonstrated that increased levels of HSP72, induced either by heat shock or by a nonheat-shock pretreatment, protected human endothelial cells against neutrophil-induced necrosis [147]. A similar study was undertaken in primary cultures of porcine endothelial cells, showing that protection against lipopolysaccharide arsenite-induced apoptosis was not only due to HSP70 upregulation but also to augmented levels of the inhibitor κB alpha and decreased NF-κB binding activity [148]. Experiments performed in cultured endothelial cells with transient overexpression of HSP70 suggested that HSP70 could be the main factor responsible for the HSR-mediated protection against LPS-induced apoptosis [149]. Similarly, Bernardini *et al.* showed that the synergistic action of HSP70, HSP32, and VEGF mediated protection against LPS-induced apoptosis in aortic endothelial cells [150]. In conclusion, *in vitro* studies highlight antioxidant, anti-inflammatory, and antiapoptotic properties of intracellular HSP70.

5.1.2.2. *In vivo.* HSP70 has been already shown to exert anti-inflammatory functions [151], by inhibiting leukocyte adhesion and recruitment [152] or by decreasing NF-κB activation and the number of activated macrophages in a model of brain inflammation of mice overexpressing HSP70 [119]. Other approaches, such as the induction of HSPs by low dose alcohol consumption, have been proposed. The authors suggested that the cardioprotective effect showed in rats was mediated by increased HSP levels, namely HSP70 and HSP32 [153].

We have shown that treatment of ApoE$^{-/-}$ mice with 17-AAG/17-DMAG upregulated HSP70 expression in the aortic arch, which was associated with

attenuated inflammation and a significant reduction in plaque size and lipid content [41]. Recently, in a mouse model of ischemia and oxidative aggression induced by severe heat stress (42 °C) for 1 h, HSP72-overexpressing mice displayed higher levels of antioxidant enzymes (glutathione peroxidase and glutathione reductase) [154]. A similar approach was used in a rat model of heat-stroke circulatory shock, in which rats were heat-shocked for 1 h at 43 °C. HSP72 expression was assessed 16 and 96 h following this 1 hour preconditioning and showed that HSP72 expression in the striatum peaked at 16 h, paralleled by reduced oxidative stress markers, whereas at 96 h, HSP72 expression was similar to that of basal levels [155].

In vitro and *in vivo* studies in the field of CVD confirm the well-described cytoprotective role of intracellular HSP70. In atherothrombosis, HSP70 could act as an intracellular shield in various cells, inhibiting different processes involved in the formation, development, and rupture of the atheromatous plaque.

5.1.3. *HSP27*

5.1.3.1. *In vitro.* Reactive Oxygen Species (ROS) represent the main trigger of protein misfolding, causing an increase in HSP expression and other protective responses (i.e., antioxidant response) [65]. Perturbations in cytoskeletal structure are one of the major consequences of extensive oxidative stress [156]. Small HSPs (i.e., HSP27) as well as other HSPs, such as HSP90 or the HSP70 families, protect intermediate filaments and microfilaments, thus preventing damage of the centrosome [6,157]. Aggregated and native LDL are both able to induce HSP27 dephosphorylation, leading to its subcellular reorganization to the tip of actin stress fibers and focal adhesion structures [110]. HSP27 was shown long ago to be involved in F-actin assembly [158] and expressed by normal medial SMCs [159].

A possible role of HSP27 in the protection against chronic inflammatory response has been suggested, due to the decreased levels of HSP27 in complicated atherosclerotic plaques [68] or unstable plaques [160]. Using two different VSMC lines, Chen *et al.* found that HSF-1 silencing by small interfering RNA (siRNA) decreased HSP27 levels. Moreover, the inflammatory response to angiotensin-II (Ang-II) was exacerbated in HSF-1 siRNA-transfected cells, suggesting a role for HSF-1 and HSP27 in the modulation of the inflammatory response [161]. Accordingly, Voegeli *et al.* later showed in VSMCs that siRNA-targeting HSP27 increased the phosphorylation of the p65 subunit of NF-κB induced by Ang-II [66]. In endothelial cells, inhibition of HSP27 phosphorylation via interference with VEGF-induced p38-MAPK signaling led to decreased actin polymerization and cell migration, indicating a potential role of HSP27 and its phosphorylation state in angiogenesis or neovascularization [162]. Recently, contrasting with this

anti-inflammatory role, it has been reported that HSP27 may participate in switching transient high activation of NF-κB into a chronic sustained activation in endothelial cells [163].

Augmented levels of HSP27 are also associated with higher levels of glutathione, which protect the cell against oxidative stress [65]. The well-known cardioprotective effect of resveratrol (an antioxidant molecule) was further studied by Wang *et al.* who found that inhibition of human aortic SMC proliferation by resveratrol was accompanied by a significant increase in HSP27 levels [164]. Another widely investigated intracellular effect of HSP27 is its antiapoptotic properties. HSP27 has been shown to inhibit the release of mitochondrial cytochrome *c* [165,166] and to inactivate cytochrome *c* by direct binding [167,168]. HSP27 has also been shown to enhance resistance to apoptosis in many other tissues [169–171]. VSMC disappearance is involved in the weakening of the fibrous cap [172], and this loss may come from disruption of extracellular survival signals by proteases, which degrade extracellular matrix components [173,174]. Anoikis, an apoptotic process subsequent to detachment, may contribute to plaque instability in atherosclerosis originating from the loss of extracellular matrix [174]. We showed that siRNA-mediated silencing of HSP27 in VSMCs treated with plasmin led to cell detachment accompanied by apoptotic features [57]. By modulating VSMC apoptosis, HSP27 could favor plaque stability.

5.1.3.2. *In vivo*. *In vivo* studies in a model of vascular remodeling induced by surgical injury to the rat carotid artery showed that when carotid levels of HSP27 peaked at 14 days, activation of NF-κB started to decrease, suggesting a possible role of HSP27 in modulating inflammation [175].

Using a mouse model overexpressing HSP27 cross-bred with ApoE−/− mice fed with a high fat diet, Rayner *et al.* [111] have reported a reduced progression of atheromatous lesions associated with increased HSP27 in serum, particularly in female mice. These authors attribute this atheroprotective effect to a possible competition of HSP27 for the uptake of atherogenic lipids (i.e., modified LDL) via the Scavenger Receptor A, demonstrated *in vitro* in macrophages. In addition, they reported that macrophages overexpressing HSP27 displayed reduced cell adhesion and migration, properties that may participate in their atheroprotective role. Recently, the same group showed that extracellular release of HSP27 involved exosomes and confirmed that atheroprotection provided by HSP27 was estrogen-dependent [176].

Thus, the intracellular effects of HSP27 have been extensively studied and include cytoskeletal stablization and protection against oxidative stress, inflammation and apoptosis, supporting its beneficial role in atherosclerosis and CV-related diseases.

5.1.4. *HSP90*

5.1.4.1. *In vitro.* Two main functions of HSP90 are related to oxidative stress; its association with nitric oxide synthases (NOS) [177] and its protection of proteasomes from oxidative insults [178]. NOS synthesize nitric oxide, which has been shown to play a protective role against oxidative stress. In macrophages and VSMCs [179–181], NO inhibits LDL oxidation, a well-described proatherogenic factor involved in endothelial dysfunction and foam cell formation [182]. HSP90 enhances eNOS activity [177] and upregulates NO synthesis, thereby inhibiting oxidation of LDL. Further, stimulation of bovine aortic endothelial cells (BAECs) with native and minimally oxidized LDL impaired the calcium-dependent association HSP90/eNOS [74]. This vascular protective feature has been extensively described *in vitro*, such as, for example, in porcine coronary artery endothelial cells [183]. In BAECs, stimulation with vascular endothelial growth factor (VEGF) promoted the association of Akt and eNOS with HSP90 which represents a scaffold favoring eNOS phosphorylation and subsequent activation by Akt [184]. HSP90 inhibitors (herbamycin or geldanamycin) were also shown to decrease estradiol-dependent eNOS activation in HUVECs [185]. In addition, inhibition of HSP90 with 17-AAG was associated with decreased endothelial migration and angiogenesis. The role of HSP90 in angiogenesis was suggested to be due to its interaction with Akt and eNOS [76].

In addition, HSP90 has been shown to protect the cells against oxidation. Aging and oxidative stress increase the levels of oxidized proteins inside the cells; the proteasome is then in charge of the clearance of damaged proteins that cannot be refolded. HSP90 may act as a shield for the 20S proteasome or multicatalytic proteinase [178,186,187], but to our knowledge there is no published data related to HSP90 and the proteasome in the field of vascular research.

However, HSP90 was shown to mediate the phosphorylation of ERK1/2, promoting its nuclear translocation, and thus increasing rat VSMC proliferation in response to oxidative stress [188]. Whether VSMC proliferation is good or bad in atherogenesis is still a matter of debate: whereas it can be considered as a healing process in response to various noxious stimuli favoring plaque stability, it can also participate in arterial wall thickening and stenosis.

HSP90 could also modulate monocyte proinflammatory response. It has been recently reported that mactinin, an inducer of monocyte maturation and present *in vivo* at sites of monocytic activation, associates with HSP90. Mactinin was found to promote production of a number of proinflammatory and chemotactic cytokines for monocytes via the inhibition of HSP90 activity [77]. In contrast, HSP90 has been shown to activate the formation of

bradykinin on endothelial cells [72] via activation of the prekallikrein–kininogen complex [189]. Bradykinin is a member of the kinin family which is a family of proinflammatory peptides involved in CVD such as atherosclerosis [190].

HSP90 may promote macrophage survival upon stimulation with oxLDL by binding to eukaryotic elongation factor-2 kinase (eEF2 kinase). Indeed, dissociation of this complex by the HSP90 inhibitor, geldanamycin, decreased the viability of macrophages [191]. Antiapoptotic effects of HSP90 were also reported by Lin *et al.* who observed that increased formation of HSP90/eNOS complexes by adiponectin protected HUVECs from Ang-II-induced apoptosis [75]. The same group found that apoptosis of HUVECs induced by high glucose, a feature of type II diabetes which is associated with a poor cardiovascular outcome, could be prevented by increased HSP90/NOS complex formation and recruitment of activated Akt [192]. Similar data regarding the antiapoptotic effects of HSP90 in association with Akt/eNOS and other client proteins in endothelial cells [193–196] and in other cell types involved in the atherosclerotic lesion have been described [197].

5.1.4.2. *In vivo.* Confirmation *in vivo* that HSP90 is a modulator of eNOS activity was shown in a model of newborn piglet pulmonary circulation [198] and in rats subjected to exercise. In the physically trained group, eNOS activity was significantly higher, as was the eNOS/HSP90 association, which could explain in part the dynamic changes in redox status following chronic exercise [199].

The largest amount of literature related to HSP90 and inflammation in CVD has arisen from the use of HSP90 inhibitors because, as mentioned before, using the HSP90 inhibitors derived from geldanamycin led to the upregulation of HSP70 whose anti-inflammatory functions have been widely demonstrated. Bucci *et al.* described a not very well-documented role of NO in acute inflammation. In a model of inflammatory response to carrageenan in eNOS-deficient mice, the authors suggested that eNOS and its interaction with HSP90 are key factors in the modulation of the vascular inflammatory response [200].

In conclusion, HSP90 is a novel player in atherosclerosis, displaying anti-apoptotic functions and a potential protective role against oxidative stress via the stimulation of eNOS activity. Its role in promoting inflammation needs to be clarified.

5.2. Extracellular Effects

Based on the idea of the heat-shock paradox [1], extracellular HSPs are viewed as a trigger for the immune response due to their potent immunogenic role and their potential function as intercellular signals. Extracellular HSPs

and their involvement in vascular diseases are gaining attention due to their importance in processes, such as inflammation, related to chronic autoimmune disease.

HSPs exit the cells to the extracellular milieu by two different mechanisms; a passive release, which usually follows prior cell damage, and active release, which involves exosomes [176,201,202] or lysosomes-like vesicles [111]. In addition, infections may represent a nonendogenous source of HSPs in the extracellular compartment as mentioned above [35–37,39,40].

5.2.1. *HSP60*

5.2.1.1. *In vitro.* To delineate the potential mechanisms whereby HSP60 is involved in atherogenesis, *in vitro* experiments were performed. Human serum anti-HSP65 antibodies act as autoantibodies reacting with HSP60 on stressed endothelial cells and are able to mediate endothelial cytotoxicity [203]. In addition to endothelial cells, macrophages expressing HSP60 can be lysed by autoantibodies against HSP65/60. Since macrophage death contributes to enlargement of the necrotic core, this effect can contribute to atherosclerotic plaque instability [204].

Further studies analyzed the potential effect of HSP60 on the mechanisms associated with atherothrombosis. Chlamydial HSP60 induced expression in vascular cells of matrix metalloproteases [205], key proteases promoting plaque rupture. In agreement, HSP60 was able to induce proinflammatory cytokines [21], possibly via the CD14 receptor [206], although it could be dependent on the cell type since in adult rat cardiomyocytes, extracellular HSP60 enhanced apoptosis via TLR-4 and completely independently of TLR-2 and CD14 [16].

5.2.1.2. *In vivo.* To address the role of HSP60/HSP65 in atherosclerosis, *in vivo* studies were performed. In the earliest study, normocholesterolemic rabbits were immunized with different antigens with/without adjuvant. Atherosclerotic lesions in the intima of the aortic arch were found to have developed only in those animals immunized with antigenic preparations containing HSP65, either as part of the whole mycobacteria or purified recombinant HSP65, in spite of their normal serum cholesterol levels. Further, combined immunization with HSP65-containing material and a cholesterol-rich diet promoted the development of significantly more severe atherosclerosis [207].

C. pneumoniae infection combined with a cholesterol-rich diet induced the development of autoantibodies against mHSP60 and this was associated with the enhanced development of lipid lesions [22]. In agreement, anti-HSP60 autoantibodies isolated from blood of patients with CHD and injected into the tail vein of apolipoprotein E-deficient mice induced the formation of atherosclerotic lesions. Further, administration of a specific mouse

monoclonal antibody to HSP60 effectively induced atherogenesis in apolipoprotein E-deficient mice [208]. Interestingly, high levels of HSP60 autoantibodies are considered to be an important prothrombotic factor that may impact cardiovascular disease [209].

Overall, extracellular HSP60 has been shown to exert a clear proatherogenic function. It has a close relationship with infection, probably due to the high homology between human and microbial HSP60/65, triggering the immune response and thus favoring a chronic state of inflammation.

5.2.2. *HSP70*

5.2.2.1. *In vitro.* Several studies have shown that HSPs, including HSP70 [210], are present in the extracellular milieu and that HSP70 can be exchanged between cells [211]. Johnson *et al.* reported that exogenous HSP70 was not internalized but remained associated with the cell surface of serum-deprived arterial SMCs, protecting the cells against noxious stimuli [212]. Also, HSP70 supplementation was shown to limit cytotoxic effects induced by serum starvation in aortic SMCs [47].

Oxidized LDLs were shown to induce HSP70 secretion by macrophages, which in turn stimulated IL-1 beta and IL-12 expression by naïve macrophages. HSP70 could therefore have a paracrine effect. OxLDL also stimulated HSP70 production by SMCs and nonconfluent proliferating endothelial cells [45,213]. In human monocytes, the dual character of HSP70 was shown to be due to its extracellular proinflammatory function via activation of NF-κB signaling and inducing proinflammatory cytokines TNF-α, IL-1β, and IL-6 [49].

Extracellular HSP70 was shown to induce proliferation of endothelial cells and tube formation *in vitro* and also promoted calcification of VSMCs. HSP70 may bind and activate Matrix Gla Protein (MGP), an inhibitor of Bone Morphogenetic Protein-4 (BMP-4). The authors hypothesized that HSP70 could be a key factor in unbalancing the positive feedback between BMP inhibitors and BMP signaling [50].

5.2.2.2. *In vivo.* In a rat model of balloon-injury, immunization with HSP70 accelerated the intimal thickening. Increased release of HSP70 within the damaged artery may produce an anti-HSP70 response and contribute to a proinflammatory state, thus suggesting that HSP70 may be a potent immunogen and explaining the accelerated neointimal formation. Also, T-cells could migrate to HSP70-rich areas and promote SMC migration and leukocyte chemoattraction via cytokine production [51].

HSP70 release into the extracellular millieu results from various stress conditions. Extracellular HSP70 seems to trigger an immune response leading to increased levels of inflammation, proliferation, and calcification. However, some studies have provided information about the antiapoptotic

role of HSP70. Therefore, further studies addressing the extracellular roles of HSP70 are neccesary to better understand its participation in atherothrombotic processes.

5.2.3. *HSP27*

Extracellular HSP27 has been suggested to play an anti-inflammatory role in atherosclerosis [111]. For example, HSP27 has been shown to induce IL-10 production by human monocytes and is thus proposed to represent an anti-inflammatory stimulus [61]. Exogenous addition of recombinant HSP27 was able to reduce spontaneous apoptosis of human neutrophils isolated from peripheral blood, without any modification of cytokine secretion (TNF-α, IL-10, IL-12) by these cells [67]. In contrast, HSP70 was reported to accelerate neutrophil apoptosis, but also reduced oxidative stress and stimulated anti-inflammatory cytokine production [62]. Rayner *et al.* suggested the development of therapies based on supplementation by exogenous HSP27 which could interact with SR-A, leading to reduced cholesterol uptake into the vessel. They conclude suggesting that "Studies testing these innovative formulations of recombinant HSP27 are underway both pre- and postinduction of atherosclerosis." [111].

5.2.4. *HSP90*

A possible role for HSP90 in modulating the extracellular activation of ERK1/2 in response to oxidative stress was proposed by Liao *et al.* in rat VSMCs [78]. Extracellular HSP90 has been recently implicated in the induction of the proatherogenic cytokine IL-8 via TLR-4 in human aortic SMCs, in addition to upregulation of IL-6, activation of ERK and p38 MAPK [79]. Also, the proinflammatory activation of the kinin-forming cascade by HSP90 in HUVECs may be extracellular in pathologic circumstances in which HSP90 is highly expressed or secreted [72]. Hence, extracellular HSP90, whose role in CVD has not been fully described, appears to be proatherogenic.

It is important to note that an intense debate exists concerning whether experimental conditions have been carefully followed using recombinant HSPs as an *in vitro* model for extracellular HSPs [214]. LPS contamination, other PAMPs (Pathogen-associated molecular patterns) or molecules bound to and/or chaperoned by HSPs might be responsible for some of their immunogenic responses. Thus, caution should be exercised when analyzing data coming from the use of recombinant HSPs.

6. HSP as Therapeutic Targets in CVD/Atherothrombosis

Since several HSPs are considered to be protective in various stress conditions, modulation of their expression has been considered as a potential therapeutic strategy in cardiovascular disease. Other potentially deleterious HSPs have been the target of immunization. It was not until the late 1980s that HSPs were examined as potential restorative molecules in cardiovascular diseases [215–217].

6.1. HSP Induction

6.1.1. *Thermal Preconditioning*

Thermal preconditioning was used in a rat model of atherosclerosis in which induction of HSPs (including HSP72 in the media) reduced not only the neointimal thickening but also the inflammatory infiltration (MCP1) and oxidative stress (p22-phox) [218]. Moreover, it has been found that moderate heating inhibited in-stent restenosis and neointimal hyperplasia in an atherosclerotic model in rabbits, and this beneficial effect has been associated with HSP70 overexpression [219]. These studies showed the importance of global HSP upregulation as a potential therapeutic strategy in CVD. Indeed, thermal therapy has been shown to improve vascular endothelial function in CVD patients [220,221]. This approach has the advantages of simplicity and feasibility. However, this strategy could potentially enhance both pro- and antiatherosclerogenic HSPs, as well as other beneficial or harmful mechanisms, which should be evaluated in detail. In any case, thermal therapy may only produce acute overexpression of certain HSPs, and its impact on a chronic disease such as atherosclerosis may thus be rather limited.

6.1.2. *Gene Therapy/Recombinant Proteins*

New approaches to overexpress HSPs such as gene therapy were carried out in mice by Rayner *et al.* [111]. Interestingly, mice overexpressing HSP27 ($ApoE^{-/-}$ $HSP27^{o/e}$) showed significantly decreased lesion size relative to $ApoE^{-/-}$ mice with normal levels of HSP27. It should be noted that female littermates displayed smaller atherosclerotic lesions than males, accompanied with a 10-fold higher HSP27 concentration in serum. The release of HSP27 was suggested to be mediated by acetylated LDL (acLDL) and estrogens, which could explain the differences found between female and male littermates. This was accompanied by a marked reduction in the secretion of the proinflammatory cytokine IL-1β and an increase in the extracellular levels of the anti-inflammatory cytokine IL-10. Moreover, HSP27 acts competitively by binding to scavenger receptor A, inhibiting thereby the

engulfment of acLDL by macrophages, a step required to acquire the foam cell phenotype. In a model of rat heart transplantation, HSP70 gene overexpression caused an improvement in ventricular and endothelial function [222] and in cardioprotection against ischemia–reperfusion injury [223].

Recombinant HSPs have also been used as therapeutic compounds in a model of mice exposed to cigarette smoke [224]. Matsumoto *et al.* injected HSP70 intravenously into mice previously exposed to cigarette smoke, known to induce intimal thickening after arterial wall damage [225,226]. HSP70-injected mice showed a decreased intimal thickening compared to controls (saline-injected mice); the authors hypothesized that prevention of intimal thickening could be mediated by the inhibition of ERK activation.

Overall, these data highlight the beneficial effects of specific upregulation of atheroprotective HSPs limiting atherosclerotic lesion development and intimal thickening associated with endothelial protection. However, suppression of proatherogenic HSP by other approaches, such as siRNAs, could also be used. In any case, to translate this approach into a clinical scenario, several parameters should be evaluated such as the safety of gene therapy, and the bio-distribution and stability of recombinant proteins or siRNAs.

6.1.3. *Pharmacological Compounds*

These promising results obtained by thermal preconditioning and gene therapy have led to the development of pharmacological compounds affecting HSP expression for treatment of different diseases, among them atherothrombosis, because of the hazards of submitting patients to a heat shock and the difficulties of using approaches such as gene therapy in humans. In 2003, Connolly *et al.* found that stimulation by Herbamycin A resulting in the production of HSP27, but not of HSP70, was responsible for decreasing intimal hyperplasia in a rat carotid ballon injury model [227]. Another pharmacological compound called YC-1, an inducer of HSP70 [46], inhibited neointima formation in a model of balloon-injured rat carotid artery [228], thus suggesting a possible therapeutic use in treatment of vascular diseases.

In an ApoE$^{-/-}$ mouse model fed a high cholesterol diet, HSP70 upregulation by using low doses of HSP90 inhibitors (17-AAG/17-DMAG) decreased inflammatory markers and also reduced aortic atherosclerotic plaque size as well as its lipid content [41]. This approach using HSP90 inhibitors has been largely used in other fields such as cancer, where it is now entering clinical trials [229–233]. Finally, statins, the most widely administered pharmacological compounds used in the treatment of CVD around the world, have shown among their numerous pleiotropic effects, the ability to increase the expression of HSPs, such as HSP90, HSP70, HSP32 (HO-1), and to activate the HSP70 promoter via the HSE, thus activating heat-shock gene transcription [234].

These latest positive data for the treatment of atherosclerosis in animal models raise the question of their use in the treatment of other CVD [235].

Thus, specific upregulation of atheroprotective HSP by pharmacological compounds could be a promising approach for the treatment of atherothrombosis. However, one important issue that should be considered is the dosage used since some of these compounds have proapoptotic actions that could have deleterious effects in terms of plaque stability. Data emanating from clinical trials using these pharmacological compounds should be examined in detail to highlight the pros and cons of their use in humans. For example, the dosage and toxicity of 17-AAG gained from phase I clinical trials in cancer patients should facilitate the evaluation of HSP90 inhibitors in non-neoplasic disorders, such as cardiovascular diseases.

6.2. Immune Therapy

As stated earlier in this review, extracellular HSPs are likely to influence atherosclerosis by triggering an immune response associated with a proinflammatory state. HSP60, due to its homology with bacterial HSP65, has given rise to a number of studies in which the immune response initiated by HSP60/HSP65 was investigated. HSP60 is now considered to be a major autoantigen in atherosclerosis [236,237]. Several approaches based on HSP60/65 immunization have been attempted in experimental models of atherosclerosis.

In 2002, Harats *et al.* [238] tested the effect of inducing mucosal tolerance to HSP60 in an atherosclerosis model accelerated by *Mycobacterium tuberculosis*. LDL receptor-deficient mice (LDL-R$^{-/-}$) were given HSP65 orally, and two different models of accelerated atherosclerosis were tested, i.e. a high fat diet or immunization with heat killed *M. tuberculosis*. In both proatherosclerotic scenarios, oral administration of HSP65 limited plaque formation. A possible explanation could be a cellular response to HSP65 leading to increased Th2 cytokine IL-4 in HSP65 immunized mice, which was previously described to possess a protective role in atherosclerosis [239]. A very similar paper by Maron *et al.* reported a decrease in atherosclerotic plaque formation in the aortic arch of LDL-R$^{-/-}$ mice having a received nasal mucosal administration of HSP65. They hypothesized that this could be related to higher levels of IL-10 and to a greater Th2 type humoral (IgG1) response in animals which received nasal administration [240]. A third study along the same line showed that oral tolerance to a small immunogenic peptide of HSP60 or to that of full-length HSP60 both reduced the plaque size by 80%. They proposed that an increase in the number of CD4+ CD25+ Foxp3+ Tregs cells could participate in the augmented production of TGF-β and IL-10 and finally contribute to decrease inflammation in the atherosclerotic area [241]. The specificity of this response is

shown by the fact that oral administration of a highly conserved sequence of HSP70 could not reduce atherosclerosis.

In 2009 Xiong *et al.* showed, in wild-type rabbits fed with a high cholesterol diet, that nasal immunization with HSP65 could attenuate atherosclerosis and reduce lipid levels [242]. Similar attempts have been made in the field of other chronic inflammatory diseases such as rheumatoid arthritis [243]. Immunization against proatherogenic HSPs could open new therapeutic avenues in cardiovascular disease although similar concerns to those commented above for gene therapy should be taken into account. The extracellular roles of the different HSPs should be further studied in order to conceive new strategies aiming at modulating HSP circulating levels (supplementation or increased clearance depending on their beneficial or deleterious role in atherogenesis).

7. Conclusions

HSP expression is modulated by different stimuli involved in all steps of atherogenesis including oxidative stress, apoptosis, proteolytic aggression, or inflammation. This could be reflected by changes in HSP protein levels in the extracellular compartment, potentially impacting their plasma levels. Also, antibodies to HSPs may be used to monitor the response to different types of stress able to induce changes in HSP levels. In the field of cardiovascular disease, although several HSPs have been suggested to be markers of the pathology, caution must be exercised since their expression may be transient, in response to an acute event, such as myocardial infarction for example, or reflecting a chronic state of inflammation or oxidative stress rather than a specific risk of future cardiovascular event. The biological roles of intracellular HSPs in atherogenesis are potentially very important since most of them protect the cells of the vasculature against various noxious stimuli. The emerging role of extracellular HSPs appears to be linked to CVD. As a consequence, therapeutic efforts for treating CVD such as atherosclerosis should take into account modulations in the recognition, binding, and internalization of HSPs. HSPs could then be considered as potential therapeutic targets to reinforce vascular defenses and delay or avoid clinical complications associated with atherothrombosis.

ACKNOWLEDGMENTS

The authors' work have been supported by the Leducq Foundation, the Spanish Ministerio de Ciencia y Tecnología (SAF 2010-21852), CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035; RD06/0014/0008), and European Network (HEALTH F2-2008-200647) and EUS2008-03565. The authors would like to thank Dr. Mary Osborne-Pellegrin for editing this chapter.

References

[1] Y. Chen, T.S. Voegeli, P.P. Liu, E.G. Noble, R.W. Currie, Heat shock paradox and a new role of heat shock proteins and their receptors as anti-inflammation targets, Inflamm. Allergy Drug Targets 6 (2007) 91 100.

[2] F. Ritossa, Discovery of the heat shock response, Cell Stress Chaperones 1 (1996) 97 98.

[3] A. Tissieres, H.K. Mitchell, U.M. Tracy, Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs, J. Mol. Biol. 84 (1974) 389 398.

[4] J.C. Ranford, B. Henderson, Chaperonins in disease: mechanisms, models, and treatments, Mol. Pathol. 55 (2002) 209 213.

[5] A. Parcellier, S. Gurbuxani, E. Schmitt, E. Solary, C. Garrido, Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways, Biochem. Biophys. Res. Commun. 304 (2003) 505 512.

[6] N. Mounier, A.P. Arrigo, Actin cytoskeleton and small heat shock proteins: how do they interact? Cell Stress Chaperones 7 (2002) 167 176.

[7] M. Ghayour-Mobarhan, A.A. Rahsepar, S. Tavallaie, S. Rahsepar, G.A. Ferns, The potential role of heat shock proteins in cardiovascular disease: evidence from in vitro and in vivo studies, Adv. Clin. Chem. 48 (2009) 27 72.

[8] D.S. Latchman, Heat shock proteins and cardiac protection, Cardiovasc. Res. 51 (2001) 637 646.

[9] D.B. DeFranco, L. Ho, E. Falke, C.W. Callaway, Small molecule activators of the heat shock response and neuroprotection from stroke, Curr. Atheroscler. Rep. 6 (2004) 295 300.

[10] H.H. Kampinga, J. Hageman, M.J. Vos, H. Kubota, R.M. Tanguay, E.A. Bruford, et al., Guidelines for the nomenclature of the human heat shock proteins, Cell Stress Chaperones 14 (2009) 105 111.

[11] X. Lu, V. Kakkar, The role of heat shock protein (HSP) in atherosclerosis: pathophysiology and clinical opportunities, Curr. Med. Chem. 17 (2010) 957 973.

[12] A. Amberger, C. Maczek, G. Jurgens, D. Michaelis, G. Schett, K. Trieb, et al., Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins, Cell Stress Chaperones 2 (1997) 94 103.

[13] Z.X. Shan, Q.X. Lin, C.Y. Deng, J.N. Zhu, L.P. Mai, J.L. Liu, et al., miR-1/miR-206 regulate Hsp60 expression contributing to glucose-mediated apoptosis in cardiomyocytes, FEBS Lett. 584 (2010) 3592 3600.

[14] R. Kleindienst, Q. Xu, J. Willeit, F.R. Waldenberger, S. Weimann, G. Wick, Immunology of atherosclerosis. Demonstration of heat shock protein 60 expression and T lymphocytes bearing alpha/beta or gamma/delta receptor in human atherosclerotic lesions, Am. J. Pathol. 142 (1993) 1927 1937.

[15] D. Chandra, G. Choy, D.G. Tang, Cytosolic accumulation of HSP60 during apoptosis with or without apparent mitochondrial release: evidence that its pro-apoptotic or pro-survival functions involve differential interactions with caspase-3, J. Biol. Chem. 282 (2007) 31289 31301.

[16] S.C. Kim, J.P. Stice, L. Chen, J.S. Jung, S. Gupta, Y. Wang, et al., Extracellular heat shock protein 60, cardiac myocytes, and apoptosis, Circ. Res. 105 (2009) 1186 1195.

[17] C.C. Deocaris, S.C. Kaul, R. Wadhwa, On the brotherhood of the mitochondrial chaperones mortalin and heat shock protein 60, Cell Stress Chaperones 11 (2006) 116 128.

[18] A.M. Czarnecka, C. Campanella, G. Zummo, F. Cappello, Heat shock protein 10 and signal transduction: a "capsula eburnea" of carcinogenesis? Cell Stress Chaperones 11 (2006) 287 294.

[19] L.H. Sigal, S. Williams, B. Soltys, R. Gupta, H9724, a monoclonal antibody to Borrelia burgdorferi's flagellin, binds to heat shock protein 60 (HSP60) within live neuroblastoma cells: a potential role for HSP60 in peptide hormone signaling and in an autoimmune pathogenesis of the neuropathy of Lyme disease, Cell. Mol. Neurobiol. 21 (2001) 477 495.
[20] S. Hirono, E. Dibrov, C. Hurtado, A. Kostenuk, R. Ducas, G.N. Pierce, Chlamydia pneumoniae stimulates proliferation of vascular smooth muscle cells through induction of endogenous heat shock protein 60, Circ. Res. 93 (2003) 710 716.
[21] W. Chen, U. Syldath, K. Bellmann, V. Burkart, H. Kolb, Human 60-kDa heat-shock protein: a danger signal to the innate immune system, J. Immunol. 162 (1999) 3212 3219.
[22] L. Erkkila, K. Laitinen, K. Haasio, T. Tiirola, M. Jauhiainen, H.A. Lehr, et al., Heat shock protein 60 autoimmunity and early lipid lesions in cholesterol-fed C57BL/6JBom mice during Chlamydia pneumoniae infection, Atherosclerosis 177 (2004) 321 328.
[23] Q. Xu, G. Schett, H. Perschinka, M. Mayr, G. Egger, F. Oberhollenzer, et al., Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population, Circulation 102 (2000) 14 20.
[24] A.G. Pockley, R. Wu, C. Lemne, R. Kiessling, F.U. de, J. Frostegard, Circulating heat shock protein 60 is associated with early cardiovascular disease, Hypertension 36 (2000) 303 307.
[25] K. Mandal, A.R. Afzal, S.J. Brecker, J. Poloniecki, Q. Xu, M. Jahangiri, Association of serum soluble heat shock protein 60 with toll-like receptor 4 polymorphism and severity of coronary artery disease, Heart 92 (2006) 683 685.
[26] X. Zhang, M. He, L. Cheng, Y. Chen, L. Zhou, H. Zeng, et al., Elevated heat shock protein 60 levels are associated with higher risk of coronary heart disease in Chinese, Circulation 118 (2008) 2687 2693.
[27] D. Rothenbacher, A. Hoffmeister, G. Bode, M. Miller, W. Koenig, H. Brenner, Helicobacter pylori heat shock protein 60 and risk of coronary heart disease: a case control study with focus on markers of systemic inflammation and lipids, Atherosclerosis 156 (2001) 193 199.
[28] J. Lewthwaite, N. Owen, A. Coates, B. Henderson, A. Steptoe, Circulating human heat shock protein 60 in the plasma of British civil servants: relationship to physiological and psychosocial stress, Circulation 106 (2002) 196 201.
[29] G. Schett, B. Metzler, R. Kleindienst, A. Amberger, H. Recheis, Q. Xu, et al., Myocardial injury leads to a release of heat shock protein (hsp) 60 and a suppression of the anti-hsp65 immune response, Cardiovasc. Res. 42 (1999) 685 695.
[30] Q. Xu, S. Kiechl, M. Mayr, B. Metzler, G. Egger, F. Oberhollenzer, et al., Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis: clinical significance determined in a follow-up study, Circulation 100 (1999) 1169 1174.
[31] J. Zhu, A.A. Quyyumi, D. Rott, G. Csako, H. Wu, J. Halcox, et al., Antibodies to human heat-shock protein 60 are associated with the presence and severity of coronary artery disease: evidence for an autoimmune component of atherogenesis, Circulation 103 (2001) 1071 1075.
[32] F. Hoppichler, M. Lechleitner, C. Traweger, G. Schett, A. Dzien, W. Sturm, et al., Changes of serum antibodies to heat-shock protein 65 in coronary heart disease and acute myocardial infarction, Atherosclerosis 126 (1996) 333 338.
[33] A. Veres, G. Fust, M. Smieja, M. McQueen, A. Horvath, Q. Yi, et al., Relationship of anti-60 kDa heat shock protein and anti-cholesterol antibodies to cardiovascular events, Circulation 106 (2002) 2775 2780.
[34] H. Kervinen, T. Huittinen, O. Vaarala, M. Leinonen, P. Saikku, V. Manninen, et al., Antibodies to human heat shock protein 60, hypertension and dyslipidemia. A study of joint effects on coronary risk, Atherosclerosis 169 (2003) 339 344.

[35] M. Mayr, B. Metzler, S. Kiechl, J. Willeit, G. Schett, Q. Xu, et al., Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of Escherichia coli and Chlamydia pneumoniae: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis, Circulation 99 (1999) 1560 1566.
[36] K. Burian, Z. Kis, D. Virok, V. Endresz, Z. Prohaszka, J. Duba, et al., Independent and joint effects of antibodies to human heat-shock protein 60 and Chlamydia pneumoniae infection in the development of coronary atherosclerosis, Circulation 103 (2001) 1503 1508.
[37] K. Heltai, Z. Kis, K. Burian, V. Endresz, A. Veres, E. Ludwig, et al., Elevated antibody levels against Chlamydia pneumoniae, human HSP60 and mycobacterial HSP65 are independent risk factors in myocardial infarction and ischaemic heart disease, Atherosclerosis 173 (2004) 339 346.
[38] S. Hoshida, M. Nishino, J. Tanouchi, T. Kishimoto, Y. Yamada, Acute Chlamydia pneumoniae infection with heat-shock-protein-60-related response in patients with acute coronary syndrome, Atherosclerosis 183 (2005) 109 112.
[39] J. Zhu, R.J. Katz, A.A. Quyyumi, D.A. Canos, D. Rott, G. Csako, et al., Association of serum antibodies to heat-shock protein 65 with coronary calcification levels: suggestion of pathogen-triggered autoimmunity in early atherosclerosis, Circulation 109 (2004) 36 41.
[40] T. Huittinen, M. Leinonen, L. Tenkanen, M. Manttari, H. Virkkunen, T. Pitkanen, et al., Autoimmunity to human heat shock protein 60, Chlamydia pneumoniae infection, and inflammation in predicting coronary risk, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 431 437.
[41] J. Madrigal-Matute, O. Lopez-Franco, L.M. Blanco-Colio, B. Munoz-Garcia, P. Ramos-Mozo, L. Ortega, et al., Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis, Cardiovasc. Res. 86 (2010) 330 337.
[42] S.D. Morris, D.V. Cumming, D.S. Latchman, D.M. Yellon, Specific induction of the 70-kD heat stress proteins by the tyrosine kinase inhibitor herbimycin-A protects rat neonatal cardiomyocytes. A new pharmacological route to stress protein expression? J. Clin. Invest. 97 (1996) 706 712.
[43] Q. Xu, G. Schett, C. Li, Y. Hu, G. Wick, Mechanical stress-induced heat shock protein 70 expression in vascular smooth muscle cells is regulated by Rac and Ras small G proteins but not mitogen-activated protein kinases, Circ. Res. 86 (2000) 1122 1128.
[44] L. Ivan, F. Antohe, Hyperlipidemia induces endothelial-derived foam cells in culture, J. Recept. Signal Transduct. Res. 30 (2010) 106 114.
[45] W.M. Zhu, P. Roma, A. Pirillo, F. Pellegatta, A.L. Catapano, Oxidized LDL induce hsp70 expression in human smooth muscle cells, FEBS Lett. 372 (1995) 1 5.
[46] Y.N. Liu, S.L. Pan, C.Y. Peng, D.Y. Huang, J.H. Guh, S.C. Kuo, et al., YC-1 induces heat shock protein 70 expression and prevents oxidized LDL-mediated apoptosis in vascular smooth muscle cells, Shock 30 (2008) 274 279.
[47] A.D. Johnson, P.A. Berberian, M. Tytell, M.G. Bond, Differential distribution of 70-kD heat shock protein in atherosclerosis. Its potential role in arterial SMC survival, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 27 36.
[48] R.R. Gill, C.J. Gbur Jr., B.J. Fisher, M.L. Hess, A.A. Fowler III, R.C. Kukreja, et al., Heat shock provides delayed protection against oxidative injury in cultured human umbilical vein endothelial cells, J. Mol. Cell. Cardiol. 30 (1998) 2739 2749.
[49] A. Asea, S.K. Kraeft, E.A. Kurt-Jones, M.A. Stevenson, L.B. Chen, R.W. Finberg, et al., HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine, Nat. Med. 6 (2000) 435 442.
[50] Y. Yao, A.D. Watson, S. Ji, K.I. Bostrom, Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix Gla protein, Circ. Res. 105 (2009) 575 584.

[51] J. George, S. Greenberg, I. Barshack, M. Singh, S. Pri-Chen, S. Laniado, et al., Accelerated intimal thickening in carotid arteries of balloon-injured rats after immunization against heat shock protein 70, J. Am. Coll. Cardiol. 38 (2001) 1564 1569.
[52] A.G. Pockley, A. Georgiades, T. Thulin, F.U. de, J. Frostegard, Serum heat shock protein 70 levels predict the development of atherosclerosis in subjects with established hypertension, Hypertension 42 (2003) 235 238.
[53] J. Zhu, A.A. Quyyumi, H. Wu, G. Csako, D. Rott, A. Zalles-Ganley, et al., Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1055 1059.
[54] J.L. Martin-Ventura, A. Leclercq, L.M. Blanco-Colio, J. Egido, P. Rossignol, O. Meilhac, et al., Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation, Atherosclerosis 194 (2007) 334 341.
[55] X. Zhang, Z. Xu, L. Zhou, Y. Chen, M. He, L. Cheng, et al., Plasma levels of Hsp70 and anti-Hsp70 antibody predict risk of acute coronary syndrome, Cell Stress Chaperones 15 (2010) 675 686.
[56] I. Herz, R. Rosso, A. Roth, G. Keren, J. George, Serum levels of anti heat shock protein 70 antibodies in patients with stable and unstable angina pectoris, Acute Card. Care 8 (2006) 46 50.
[57] J.L. Martin-Ventura, V. Nicolas, X. Houard, L.M. Blanco-Colio, A. Leclercq, J. Egido, et al., Biological significance of decreased HSP27 in human atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 1337 1343.
[58] L. Santell, N.S. Bartfeld, E.G. Levin, Identification of a protein transiently phosphorylated by activators of endothelial cell function as the heat-shock protein HSP27. A possible role for protein kinase C, Biochem. J. 284 (Pt 3) (1992) 705 710.
[59] J.L. Martin, E. Hickey, L.A. Weber, W.H. Dillmann, R. Mestril, Influence of phosphorylation and oligomerization on the protective role of the small heat shock protein 27 in rat adult cardiomyocytes, Gene Expr. 7 (1999) 349 355.
[60] J.L. Martin, R. Mestril, R. Hilal-Dandan, L.L. Brunton, W.H. Dillmann, Small heat shock proteins and protection against ischemic injury in cardiac myocytes, Circulation 96 (1997) 4343 4348.
[61] A.K. De, K.M. Kodys, B.S. Yeh, C. Miller-Graziano, Exaggerated human monocyte IL-10 concomitant to minimal TNF-alpha induction by heat-shock protein 27 (Hsp27) suggests Hsp27 is primarily an antiinflammatory stimulus, J. Immunol. 165 (2000) 3951 3958.
[62] T.E. Callahan, J. Marins, W.J. Welch, J.K. Horn, Heat shock attenuates oxidation and accelerates apoptosis in human neutrophils, J. Surg. Res. 85 (1999) 317 322.
[63] M. Zhou, H. Lambert, J. Landry, Transient activation of a distinct serine protein kinase is responsible for 27-kDa heat shock protein phosphorylation in mitogen-stimulated and heat-shocked cells, J. Biol. Chem. 268 (1993) 35 43.
[64] S. Salinthone, M. Tyagi, W.T. Gerthoffer, Small heat shock proteins in smooth muscle, Pharmacol. Ther. 119 (2008) 44 54.
[65] X. Preville, F. Salvemini, S. Giraud, S. Chaufour, C. Paul, G. Stepien, et al., Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery, Exp. Cell Res. 247 (1999) 61 78.
[66] T.S. Voegeli, R.W. Currie, siRNA knocks down Hsp27 and increases angiotensin II-induced phosphorylated NF-kappaB p65 levels in aortic smooth muscle cells, Inflamm. Res. 58 (2009) 336 343.
[67] K. Sheth, A. De, B. Nolan, J. Friel, A. Duffy, R. Ricciardi, et al., Heat shock protein 27 inhibits apoptosis in human neutrophils, J. Surg. Res. 99 (2001) 129 133.

[68] J.L. Martin-Ventura, M.C. Duran, L.M. Blanco-Colio, O. Meilhac, A. Leclercq, J.B. Michel, et al., Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis, Circulation 110 (2004) 2216 2219.
[69] B. Dybdahl, S.A. Slordahl, A. Waage, P. Kierulf, T. Espevik, A. Sundan, Myocardial ischaemia and the inflammatory response: release of heat shock protein 70 after myocardial infarction, Heart 91 (2005) 299 304.
[70] M. Ghayour-Mobarhan, A. Sahebkar, S.M. Parizadeh, M. Moohebati, S. Tavallaie, S.M. Rezakazemi-Bajestani, et al., Antibody titres to heat shock protein 27 are elevated in patients with acute coronary syndrome, Int. J. Exp. Pathol. 89 (2008) 209 215.
[71] S. Shams, S. Shafi, K. Bodman-Smith, P. Williams, S. Mehta, G.A. Ferns, Anti-heat shock protein-27 (Hsp-27) antibody levels in patients with chest pain: association with established cardiovascular risk factors, Clin. Chim. Acta 395 (2008) 42 46.
[72] K. Joseph, B.G. Tholanikunnel, A.P. Kaplan, Activation of the bradykinin-forming cascade on endothelial cells: a role for heat shock protein 90, Int. Immunopharmacol. 2 (2002) 1851 1859.
[73] H. Huang, W.C. Lee, J.H. Lin, S.C. Jian, S.J. Mao, P.C. Yang, et al., Molecular cloning and characterization of porcine cDNA encoding a 90-kDa heat shock protein and its expression following hyperthermia, Gene 226 (1999) 307 315.
[74] D.W. Stepp, J. Ou, A.W. Ackerman, S. Welak, D. Klick, K.A. Pritchard Jr., Native LDL and minimally oxidized LDL differentially regulate superoxide anion in vascular endothelium in situ, Am. J. Physiol. Heart Circ. Physiol. 283 (2002) H750 H759.
[75] L.Y. Lin, C.Y. Lin, T.C. Su, C.S. Liau, Angiotensin II-induced apoptosis in human endothelial cells is inhibited by adiponectin through restoration of the association between endothelial nitric oxide synthase and heat shock protein 90, FEBS Lett. 574 (2004) 106 110.
[76] J. Sun, J.K. Liao, Induction of angiogenesis by heat shock protein 90 mediated by protein kinase Akt and endothelial nitric oxide synthase, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 2238 2244.
[77] S.D. Luikart, A. Panoskaltsis-Mortari, T. Hinkel, R.T. Perri, K. Gupta, T.R. Oegema, et al., Mactinin, a fragment of cytoskeletal alpha-actinin, is a novel inducer of heat shock protein (Hsp)-90 mediated monocyte activation, BMC Cell Biol. 10 (2009) 60.
[78] D.F. Liao, Z.G. Jin, A.S. Baas, G. Daum, S.P. Gygi, R. Aebersold, et al., Purification and identification of secreted oxidative stress-induced factors from vascular smooth muscle cells, J. Biol. Chem. 275 (2000) 189 196.
[79] S.W. Chung, J.H. Lee, K.H. Choi, Y.C. Park, S.K. Eo, B.Y. Rhim, et al., Extracellular heat shock protein 90 induces interleukin-8 in vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 378 (2009) 444 449.
[80] Y. Shi, D.D. Mosser, R.I. Morimoto, Molecular chaperones as HSF1-specific transcriptional repressors, Genes Dev. 12 (1998) 654 666.
[81] R. Businaro, E. Profumo, A. Tagliani, B. Buttari, S. Leone, G. D'Amati, et al., Heat-shock protein 90: a novel autoantigen in human carotid atherosclerosis, Atherosclerosis 207 (2009) 74 83.
[82] I.J. Benjamin, D.R. McMillan, Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease, Circ. Res. 83 (1998) 117 132.
[83] J. Galea-Lauri, A.J. Richardson, D.S. Latchman, D.R. Katz, Increased heat shock protein 90 (hsp90) expression leads to increased apoptosis in the monoblastoid cell line U937 following induction with TNF-alpha and cycloheximide: a possible role in immunopathology, J. Immunol. 157 (1996) 4109 4118.

[84] A. Dupont, M. Chwastyniak, O. Beseme, A.L. Guihot, H. Drobecq, P. Amouyel, et al., Application of saturation dye 2D-DIGE proteomics to characterize proteins modulated by oxidized low density lipoprotein treatment of human macrophages, J. Proteome Res. 7 (2008) 3572 3582.
[85] M. Clerget, B.S. Polla, Erythrophagocytosis induces heat shock protein synthesis by human monocytes-macrophages, Proc. Natl. Acad. Sci. USA 87 (1990) 1081 1085.
[86] P. Matz, P. Weinstein, B. States, J. Honkaniemi, F.R. Sharp, Subarachnoid injections of lysed blood induce the hsp70 stress gene and produce DNA fragmentation in focal areas of the rat brain, Stroke 27 (1996) 504 512.
[87] W. Palinski, C. Napoli, The fetal origins of atherosclerosis: maternal hypercholesterolemia, and cholesterol-lowering or antioxidant treatment during pregnancy influence in utero programming and postnatal susceptibility to atherogenesis, FASEB J. 16 (2002) 1348 1360.
[88] H.F. Chen, L.D. Xie, C.S. Xu, Role of heat shock protein 27 phosphorylation in migration of vascular smooth muscle cells, Mol. Cell. Biochem. 327 (2009) 1 6.
[89] N. Vanmuylder, L. Evrard, N. Dourov, Strong expression of heat shock proteins in growth plate cartilage, an immunohistochemical study of HSP28, HSP70 and HSP110, Anat. Embryol. (Berl) 195 (1997) 359 362.
[90] W.E. Hellings, W. Peeters, F.L. Moll, S.R. Piers, S.J. van, P.J. Van der Spek, et al., Composition of carotid atherosclerotic plaque is associated with cardiovascular outcome: a prognostic study, Circulation 121 (2010) 1941 1950.
[91] Q. Xiao, K. Mandal, G. Schett, M. Mayr, G. Wick, F. Oberhollenzer, et al., Association of serum-soluble heat shock protein 60 with carotid atherosclerosis: clinical significance determined in a follow-up study, Stroke 36 (2005) 2571 2576.
[92] Q. Xu, R. Kleindienst, W. Waitz, H. Dietrich, G. Wick, Increased expression of heat shock protein 65 coincides with a population of infiltrating T lymphocytes in atherosclerotic lesions of rabbits specifically responding to heat shock protein 65, J. Clin. Invest. 91 (1993) 2693 2702.
[93] Q. Xu, G. Luef, S. Weimann, R.S. Gupta, H. Wolf, G. Wick, Staining of endothelial cells and macrophages in atherosclerotic lesions with human heat-shock protein-reactive antisera, Arterioscler. Thromb. 13 (1993) 1763 1769.
[94] A. Kol, T. Bourcier, A.H. Lichtman, P. Libby, Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages, J. Clin. Invest. 103 (1999) 571 577.
[95] A.G. Pockley, J. Shepherd, J.M. Corton, Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals, Immunol. Invest. 27 (1998) 367 377.
[96] J.P. Leger, F.M. Smith, R.W. Currie, Confocal microscopic localization of constitutive and heat shock-induced proteins HSP70 and HSP27 in the rat heart, Circulation 102 (2000) 1703 1709.
[97] P.A. Berberian, W. Myers, M. Tytell, V. Challa, M.G. Bond, Immunohistochemical localization of heat shock protein-70 in normal-appearing and atherosclerotic specimens of human arteries, Am. J. Pathol. 136 (1990) 71 80.
[98] I. Kardys, N. Rifai, O. Meilhac, J.B. Michel, J.L. Martin-Ventura, J.E. Buring, et al., Plasma concentration of heat shock protein 27 and risk of cardiovascular disease: a prospective, nested case-control study, Clin. Chem. 54 (2008) 139 146.
[99] H.K. Park, E.C. Park, S.W. Bae, M.Y. Park, S.W. Kim, H.S. Yoo, et al., Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome, Circulation 114 (2006) 886 893.

[100] R. Rajaiah, K.D. Moudgil, Heat-shock proteins can promote as well as regulate autoimmunity, Autoimmun. Rev. 8 (2009) 388 393.
[101] H. Perschinka, M. Mayr, G. Millonig, C. Mayerl, R. van der Zee, S.G. Morrison, et al., Cross-reactive B-cell epitopes of microbial and human heat shock protein 60/65 in atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1060 1065.
[102] X. Zhang, M.A. He, L. Cheng, L. Zhou, H. Zeng, J. Wang, et al., Joint effects of antibody to heat shock protein 60, hypertension, and diabetes on risk of coronary heart disease in Chinese, Clin. Chem. 54 (2008) 1046 1052.
[103] E. Fehrenbach, H. Northoff, Free radicals, exercise, apoptosis, and heat shock proteins, Exerc. Immunol. Rev. 7 (2001) 66 89.
[104] E.G. Noble, K.J. Milne, C.W. Melling, Heat shock proteins and exercise: a primer, Appl. Physiol. Nutr. Metab. 33 (2008) 1050 1065.
[105] M. Yang, T. Wu, L. Cheng, F. Wang, Q. Wei, R.M. Tanguay, Plasma antibodies against heat shock protein 70 correlate with the incidence and severity of asthma in a Chinese population, Respir. Res. 6 (2005) 18.
[106] G. Fust, Z. Beck, D. Banhegyi, J. Kocsis, A. Biro, Z. Prohaszka, Antibodies against heat shock proteins and cholesterol in HIV infection, Mol. Immunol. 42 (2005) 79 85.
[107] A. Figueredo, J.L. Ibarra, A. Rodriguez, A.M. Molino, Gomez-de la Concha E, Fernandez-Cruz A, et al. Increased serum levels of IgA antibodies to hsp70 protein in patients with diabetes mellitus: their relationship with vascular complications, Clin. Immunol. Immunopathol. 79 (1996) 252 255.
[108] A. Olejek, A. Damasiewicz-Bodzek, P. Bodzek, T. Wielkoszynski, J. Zamlynski, P. Stoltny, et al., Concentrations of antibodies against heat shock protein 27 in the sera of women with ovarian carcinoma, Int. J. Gynecol. Cancer 19 (2009) 1516 1520.
[109] R.T. Demmer, M. Desvarieux, Periodontal infections and cardiovascular disease: the heart of the matter, J. Am. Dent. Assoc. 137 (Suppl.) (2006) 14S 20S.
[110] M. Garcia-Arguinzonis, T. Padro, R. Lugano, V. Llorente-Cortes, L. Badimon, Low-density lipoproteins induce heat shock protein 27 dephosphorylation, oligomerization, and subcellular relocalization in human vascular smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1212 1219.
[111] K. Rayner, Y.X. Chen, M. McNulty, T. Simard, X. Zhao, D.J. Wells, et al., Extracellular release of the atheroprotective heat shock protein 27 is mediated by estrogen and competitively inhibits acLDL binding to scavenger receptor-A, Circ. Res. 103 (2008) 133 141.
[112] C. Zanini, F. Pulera, F. Carta, G. Giribaldi, G. Mandili, M.M. Maule, et al., Proteomic identification of heat shock protein 27 as a differentiation and prognostic marker in neuroblastoma but not in Ewing's sarcoma, Virchows Arch. 452 (2008) 157 167.
[113] H. Pei, H. Zhu, S. Zeng, Y. Li, H. Yang, L. Shen, et al., Proteome analysis and tissue microarray for profiling protein markers associated with lymph node metastasis in colorectal cancer, J. Proteome Res. 6 (2007) 2495 2501.
[114] R. Langer, K. Ott, K. Specht, K. Becker, F. Lordick, M. Burian, et al., Protein expression profiling in esophageal adenocarcinoma patients indicates association of heat-shock protein 27 expression and chemotherapy response, Clin. Cancer Res. 14 (2008) 8279 8287.
[115] D.L. Feinstein, E. Galea, D.A. Aquino, G.C. Li, H. Xu, D.J. Reis, Heat shock protein 70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NFkappaB activation, J. Biol. Chem. 271 (1996) 17724 17732.
[116] M.W. Van, B. Wielockx, T. Mahieu, M. Takada, T. Taniguchi, K. Sekikawa, et al., HSP70 protects against TNF-induced lethal inflammatory shock, Immunity 16 (2002) 685 695.
[117] A. Ialenti, M.P. Di, F. D'Acquisto, B. Pisano, P. Maffia, G. Grassia, et al., Inhibition of cyclooxygenase-2 gene expression by the heat shock response in J774 murine macrophages, Eur. J. Pharmacol. 509 (2005) 89 96.

[118] S.K. Jo, G.J. Ko, C.S. Boo, W.Y. Cho, H.K. Kim, Heat preconditioning attenuates renal injury in ischemic ARF in rats: role of heat-shock protein 70 on NF-kappaB-mediated inflammation and on tubular cell injury, J. Am. Soc. Nephrol. 17 (2006) 3082 3092.
[119] Z. Zheng, J.Y. Kim, H. Ma, J.E. Lee, M.A. Yenari, Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke, J. Cereb. Blood Flow Metab. 28 (2008) 53 63.
[120] U. Zugel, S.H. Kaufmann, Immune response against heat shock proteins in infectious diseases, Immunobiology 201 (1999) 22 35.
[121] E.W. van, R. van der Zee, B. Prakken, Heat-shock proteins induce T-cell regulation of chronic inflammation, Nat. Rev. Immunol. 5 (2005) 318 330.
[122] J. Ananthan, A.L. Goldberg, R. Voellmy, Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes, Science 232 (1986) 522 524.
[123] A. Ali, S. Bharadwaj, R. O'Carroll, N. Ovsenek, HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes, Mol. Cell. Biol. 18 (1998) 4949 4960.
[124] D.F. Smith, W.P. Sullivan, T.N. Marion, K. Zaitsu, B. Madden, D.J. McCormick, et al., Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70, Mol. Cell. Biol. 13 (1993) 869 876.
[125] J. Hohfeld, Y. Minami, F.U. Hartl, Hip, a novel cochaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle, Cell 83 (1995) 589 598.
[126] Y. Minami, J. Hohfeld, K. Ohtsuka, F.U. Hartl, Regulation of the heat-shock protein 70 reaction cycle by the mammalian DnaJ homolog, Hsp40, J. Biol. Chem. 271 (1996) 19617 19624.
[127] K.A. Hutchison, L.F. Stancato, J.K. Owens-Grillo, J.L. Johnson, P. Krishna, D.O. Toft, et al., The 23-kDa acidic protein in reticulocyte lysate is the weakly bound component of the hsp foldosome that is required for assembly of the glucocorticoid receptor into a functional heterocomplex with hsp90, J. Biol. Chem. 270 (1995) 18841 18847.
[128] R. Baler, G. Dahl, R. Voellmy, Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1, Mol. Cell. Biol. 13 (1993) 2486 2496.
[129] K.D. Sarge, S.P. Murphy, R.I. Morimoto, Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress, Mol. Cell. Biol. 13 (1993) 1392 1407.
[130] J.T. Westwood, C. Wu, Activation of Drosophila heat shock factor: conformational change associated with a monomer-to-trimer transition, Mol. Cell. Biol. 13 (1993) 3481 3486.
[131] R. Shringarpure, T. Grune, J. Mehlhase, K.J. Davies, Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome, J. Biol. Chem. 278 (2003) 311 318.
[132] Y.H. Lee, B. Govinda, J.C. Kim, T.I. Kim, N.H. Lee, J.C. Lee, et al., Oxidative stress resistance through blocking Hsp60 translocation followed by SAPK/JNK inhibition in aged human diploid fibroblasts, Cell Biochem. Funct. 27 (2009) 35 39.
[133] Y.H. Lee, J.C. Lee, H.J. Moon, J.E. Jung, M. Sharma, B.H. Park, et al., Differential effect of oxidative stress on the apoptosis of early and late passage human diploid fibroblasts: implication of heat shock protein 60, Cell Biochem. Funct. 26 (2008) 502 508.
[134] Q. Xu, G. Schett, C.S. Seitz, Y. Hu, R.S. Gupta, G. Wick, Surface staining and cytotoxic activity of heat-shock protein 60 antibody in stressed aortic endothelial cells, Circ. Res. 75 (1994) 1078 1085.

[135] C. Bason, R. Corrocher, C. Lunardi, P. Puccetti, O. Olivieri, D. Girelli, et al., Interaction of antibodies against cytomegalovirus with heat-shock protein 60 in pathogenesis of atherosclerosis, Lancet 362 (2003) 1971 1977.
[136] B.S. Polla, S. Kantengwa, D. Francois, S. Salvioli, C. Franceschi, C. Marsac, et al., Mitochondria are selective targets for the protective effects of heat shock against oxidative injury, Proc. Natl. Acad. Sci. USA 93 (1996) 6458 6463.
[137] L. Xu, R.G. Giffard, HSP70 protects murine astrocytes from glucose deprivation injury, Neurosci. Lett. 224 (1997) 9 12.
[138] M.R. Jacquier-Sarlin, K. Fuller, A.T. Dinh-Xuan, M.J. Richard, B.S. Polla, Protective effects of hsp70 in inflammation, Experientia 50 (1994) 1031 1038.
[139] K.M. Stuhlmeier, Activation and regulation of Hsp32 and Hsp70, Eur. J. Biochem. 267 (2000) 1161 1167.
[140] P. Pandey, A. Saleh, A. Nakazawa, S. Kumar, S.M. Srinivasula, V. Kumar, et al., Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90, EMBO J. 19 (2000) 4310 4322.
[141] H.M. Beere, B.B. Wolf, K. Cain, D.D. Mosser, A. Mahboubi, T. Kuwana, et al., Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome, Nat. Cell Biol. 2 (2000) 469 475.
[142] A. Saleh, S.M. Srinivasula, L. Balkir, P.D. Robbins, E.S. Alnemri, Negative regulation of the Apaf-1 apoptosome by Hsp70, Nat. Cell Biol. 2 (2000) 476 483.
[143] C.Y. Li, J.S. Lee, Y.G. Ko, J.I. Kim, J.S. Seo, Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation, J. Biol. Chem. 275 (2000) 25665 25671.
[144] Y. Matsumori, F.J. Northington, S.M. Hong, T. Kayama, R.A. Sheldon, Z.S. Vexler, et al., Reduction of caspase-8 and-9 cleavage is associated with increased c-FLIP and increased binding of Apaf-1 and Hsp70 after neonatal hypoxic/ischemic injury in mice overexpressing Hsp70, Stroke 37 (2006) 507 512.
[145] L. Ravagnan, S. Gurbuxani, S.A. Susin, C. Maisse, E. Daugas, N. Zamzami, et al., Heat-shock protein 70 antagonizes apoptosis-inducing factor, Nat. Cell Biol. 3 (2001) 839 843.
[146] F. Alcocer, D. Whitley, J. Salazar, W. Jordan, K.I. Bland, Mutual exclusion of apoptosis and hsp70 in human vein intimal hyperplasia in vitro, J. Surg. Res. 96 (2001) 75 80.
[147] J.H. Wang, H.P. Redmond, R.W. Watson, C. Condron, D. Bouchier-Hayes, Induction of heat shock protein 72 prevents neutrophil-mediated human endothelial cell necrosis, Arch. Surg. 130 (1995) 1260 1265.
[148] S.L. DeMeester, T.G. Buchman, Y. Qiu, A.K. Jacob, K. Dunnigan, R.S. Hotchkiss, et al., Heat shock induces IkappaB-alpha and prevents stress-induced endothelial cell apoptosis, Arch. Surg. 132 (1997) 1283 1287.
[149] H.R. Wong, R.J. Mannix, J.M. Rusnak, A. Boota, H. Zar, S.C. Watkins, et al., The heat-shock response attenuates lipopolysaccharide-mediated apoptosis in cultured sheep pulmonary artery endothelial cells, Am. J. Respir. Cell Mol. Biol. 15 (1996) 745 751.
[150] C. Bernardini, A. Zannoni, M.E. Turba, P. Fantinati, C. Tamanini, M.L. Bacci, et al., Heat shock protein 70, heat shock protein 32, and vascular endothelial growth factor production and their effects on lipopolysaccharide-induced apoptosis in porcine aortic endothelial cells, Cell Stress Chaperones 10 (2005) 340 348.
[151] M.A. Yenari, J. Liu, Z. Zheng, Z.S. Vexler, J.E. Lee, R.G. Giffard, Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection, Ann. NY Acad. Sci. 1053 (2005) 74 83.
[152] S.D. House, P.T. Guidon Jr., G.A. Perdrizet, M. Rewinski, R. Kyriakos, R.S. Bockman, et al., Effects of heat shock, stannous chloride, and gallium nitrate on the rat inflammatory response, Cell Stress Chaperones 6 (2001) 164 171.

[153] M. Sato, C. Fraga, D.K. Das, Induction of the expression of cardioprotective proteins after mild-to-moderate consumption of alcohol, Pathophysiology 10 (2004) 139 145.
[154] Z.C. Chen, W.S. Wu, M.T. Lin, C.C. Hsu, Protective effect of transgenic expression of porcine heat shock protein 70 on hypothalamic ischemic and oxidative damage in a mouse model of heatstroke, BMC Neurosci. 10 (2009) 111.
[155] J.L. Wang, D.S. Ke, M.T. Lin, Heat shock pretreatment may protect against heatstroke-induced circulatory shock and cerebral ischemia by reducing oxidative stress and energy depletion, Shock 23 (2005) 161 167.
[156] F. Mirabelli, A. Salis, M. Perotti, F. Taddei, G. Bellomo, S. Orrenius, Alterations of surface morphology caused by the metabolism of menadione in mammalian cells are associated with the oxidation of critical sulfhydryl groups in cytoskeletal proteins, Biochem. Pharmacol. 37 (1988) 3423 3427.
[157] P. Liang, T.H. MacRae, Molecular chaperones and the cytoskeleton, J. Cell Sci. 110 (Pt 13) (1997) 1431 1440.
[158] T. Miron, K. Vancompernolle, J. Vandekerckhove, M. Wilchek, B. Geiger, A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein, J. Cell Biol. 114 (1991) 255 261.
[159] D. Campisi, M. Cutolo, G. Carruba, C.M. Lo, L. Comito, O.M. Granata, et al., Evidence for soluble and nuclear site I binding of estrogens in human aorta, Atherosclerosis 103 (1993) 267 277.
[160] A.J. Lepedda, A. Cigliano, G.M. Cherchi, R. Spirito, M. Maggioni, F. Carta, et al., A proteomic approach to differentiate histologically classified stable and unstable plaques from human carotid arteries, Atherosclerosis 203 (2009) 112 118.
[161] Y. Chen, R.W. Currie, Small interfering RNA knocks down heat shock factor-1 (HSF-1) and exacerbates pro-inflammatory activation of NF-kappaB and AP-1 in vascular smooth muscle cells, Cardiovasc. Res. 69 (2006) 66 75.
[162] S. Rousseau, F. Houle, J. Landry, J. Huot, p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells, Oncogene 15 (1997) 2169 2177.
[163] M.M. Gorska, Q. Liang, S.J. Stafford, N. Goplen, N. Dharajiya, L. Guo, et al., MK2 controls the level of negative feedback in the NF-kappaB pathway and is essential for vascular permeability and airway inflammation, J. Exp. Med. 204 (2007) 1637 1652.
[164] Z. Wang, Y. Chen, N. Labinskyy, T.C. Hsieh, Z. Ungvari, J.M. Wu, Regulation of proliferation and gene expression in cultured human aortic smooth muscle cells by resveratrol and standardized grape extracts, Biochem. Biophys. Res. Commun. 346 (2006) 367 376.
[165] A.M. Gorman, E. Szegezdi, D.J. Quigney, A. Samali, Hsp27 inhibits 6-hydroxydopamine-induced cytochrome c release and apoptosis in PC12 cells, Biochem. Biophys. Res. Commun. 327 (2005) 801 810.
[166] H. Schepers, M. Geugien, M. van der Toorn, A.L. Bryantsev, H.H. Kampinga, B.J. Eggen, et al., HSP27 protects AML cells against VP-16-induced apoptosis through modulation of p38 and c-Jun, Exp. Hematol. 33 (2005) 660 670.
[167] C. Garrido, J.M. Bruey, A. Fromentin, A. Hammann, A.P. Arrigo, E. Solary, HSP27 inhibits cytochrome c-dependent activation of procaspase-9, FASEB J. 13 (1999) 2061 2070.
[168] J.M. Bruey, C. Ducasse, P. Bonniaud, L. Ravagnan, S.A. Susin, C. Diaz-Latoud, et al., Hsp27 negatively regulates cell death by interacting with cytochrome c, Nat. Cell Biol. 2 (2000) 645 652.
[169] J.E. O'Brien Jr., M.L. Ormont, Y. Shi, D. Wang, A. Zalewski, J.D. Mannion, Early injury to the media after saphenous vein grafting, Ann. Thorac. Surg. 65 (1998) 1273 1278.

[170] M. Vayssier, B.S. Polla, Heat shock proteins chaperoning life and death, Cell Stress Chaperones 3 (1998) 221 227.
[171] A. Samali, S. Orrenius, Heat shock proteins: regulators of stress response and apoptosis, Cell Stress Chaperones 3 (1998) 228 236.
[172] K. Walsh, R.C. Smith, H.S. Kim, Vascular cell apoptosis in remodeling, restenosis, and plaque rupture, Circ. Res. 87 (2000) 184 188.
[173] D.E. Ingber, Tensegrity II. How structural networks influence cellular information processing networks, J. Cell. Sci. 116 (2003) 1397 1408.
[174] J.B. Michel, Anoikis in the cardiovascular system: known and unknown extracellular mediators, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 2146 2154.
[175] B. Rinaldi, P. Romagnoli, S. Bacci, R. Carnuccio, M.C. Maiuri, M. Donniacuo, et al., Inflammatory events in a vascular remodeling model induced by surgical injury to the rat carotid artery, Br. J. Pharmacol. 147 (2006) 175 182.
[176] K. Rayner, J. Sun, Y.X. Chen, M. McNulty, T. Simard, X. Zhao, et al., Heat shock protein 27 protects against atherogenesis via an estrogen-dependent mechanism: role of selective estrogen receptor beta modulation, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 1751 1756.
[177] G. Garcia-Cardena, R. Fan, V. Shah, R. Sorrentino, G. Cirino, A. Papapetropoulos, et al., Dynamic activation of endothelial nitric oxide synthase by Hsp90, Nature 392 (1998) 821 824.
[178] M. Conconi, I. Petropoulos, I. Emod, E. Turlin, F. Biville, B. Friguet, Protection from oxidative inactivation of the 20S proteasome by heat-shock protein 90, Biochem. J. 333 (Pt 2) (1998) 407 415.
[179] N. Hogg, B. Kalyanaraman, J. Joseph, A. Struck, S. Parthasarathy, Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis, FEBS Lett. 334 (1993) 170 174.
[180] N. Hogg, A. Struck, S.P. Goss, N. Santanam, J. Joseph, S. Parthasarathy, et al., Inhibition of macrophage-dependent low density lipoprotein oxidation by nitric-oxide donors, J. Lipid Res. 36 (1995) 1756 1762.
[181] Y. Rikitake, K. Hirata, S. Kawashima, H. Akita, M. Yokoyama, Inhibitory effect of inducible type nitric oxide synthase on oxidative modification of low density lipoprotein by vascular smooth muscle cells, Atherosclerosis 136 (1998) 51 57.
[182] E.M. Morrel, J.A. Holland, K.A. Pritchard, C.K. Colton, M.B. Stemerman, Endothelial cell perturbation and low-density lipoprotein. Quantitative autoradiography, Ann. NY Acad. Sci. 516 (1987) 412 417.
[183] J.X. Chen, B. Meyrick, Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium, Lab. Invest. 84 (2004) 182 190.
[184] J. Fontana, D. Fulton, Y. Chen, T.A. Fairchild, T.J. McCabe, N. Fujita, et al., Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release, Circ. Res. 90 (2002) 866 873.
[185] K.S. Russell, M.P. Haynes, T. Caulin-Glaser, J. Rosneck, W.C. Sessa, J.R. Bender, Estrogen stimulates heat shock protein 90 binding to endothelial nitric oxide synthase in human vascular endothelial cells. Effects on calcium sensitivity and NO release, J. Biol. Chem. 275 (2000) 5026 5030.
[186] M. Conconi, B. Friguet, Proteasome inactivation upon aging and on oxidation-effect of HSP 90, Mol. Biol. Rep. 24 (1997) 45 50.
[187] M. Conconi, L.I. Szweda, R.L. Levine, E.R. Stadtman, B. Friguet, Age-related decline of rat liver multicatalytic proteinase activity and protection from oxidative inactivation by heat-shock protein 90, Arch. Biochem. Biophys. 331 (1996) 232 240.

[188] D.H. Liu, H.Y. Yuan, C.Y. Cao, Z.P. Gao, B.Y. Zhu, H.L. Huang, et al., Heat shock protein 90 acts as a molecular chaperone in late-phase activation of extracellular signal-regulated kinase 1/2 stimulated by oxidative stress in vascular smooth muscle cells, Acta Pharmacol. Sin. 28 (2007) 1907 1913.
[189] K. Joseph, B.G. Tholanikunnel, A.P. Kaplan, Heat shock protein 90 catalyzes activation of the prekallikrein-kininogen complex in the absence of factor XII, Proc. Natl. Acad. Sci. USA 99 (2002) 896 900.
[190] J. Duchene, A. Ahluwalia, The kinin B(1) receptor and inflammation: new therapeutic target for cardiovascular disease, Curr. Opin. Pharmacol. 9 (2009) 125 131.
[191] J.H. Chen, M. Riazy, E.M. Smith, C.G. Proud, U.P. Steinbrecher, V. Duronio, Oxidized LDL-mediated macrophage survival involves elongation factor-2 kinase, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 92 98.
[192] L.Y. Lin, C.Y. Lin, F.M. Ho, C.S. Liau, Up-regulation of the association between heat shock protein 90 and endothelial nitric oxide synthase prevents high glucose-induced apoptosis in human endothelial cells, J. Cell. Biochem. 94 (2005) 194 201.
[193] G.N. Antonova, C.M. Snead, A.S. Antonov, C. Dimitropoulou, R.C. Venema, J.D. Catravas, Nitric oxide preconditioning regulates endothelial monolayer integrity via the heat shock protein 90-soluble guanylate cyclase pathway, Am. J. Physiol. Heart Circ. Physiol. 292 (2007) H893 H903.
[194] J. Haendeler, J. Hoffmann, S. Rahman, A.M. Zeiher, S. Dimmeler, Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation, FEBS Lett. 536 (2003) 180 186.
[195] R. Zhang, D. Luo, R. Miao, L. Bai, Q. Ge, W.C. Sessa, et al., Hsp90-Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis, Oncogene 24 (2005) 3954 3963.
[196] B.J. Davis, Z. Xie, B. Viollet, M.H. Zou, Activation of the AMP-activated kinase by antidiabetes drug metformin stimulates nitric oxide synthesis in vivo by promoting the association of heat shock protein 90 and endothelial nitric oxide synthase, Diabetes 55 (2006) 496 505.
[197] R. Rezzani, L. Rodella, C. Dessy, G. Daneau, R. Bianchi, O. Feron, Changes in Hsp90 expression determine the effects of cyclosporine A on the NO pathway in rat myocardium, FEBS Lett. 552 (2003) 125 129.
[198] J.L. Aschner, H. Zeng, M.R. Kaplowitz, Y. Zhang, J.C. Slaughter, C.D. Fike, Heat shock protein 90-eNOS interactions mature with postnatal age in the pulmonary circulation of the piglet, Am. J. Physiol. Lung Cell. Mol. Physiol. 296 (2009) L555 L564.
[199] M.B. Harris, B.M. Mitchell, S.G. Sood, R.C. Webb, R.C. Venema, Increased nitric oxide synthase activity and Hsp90 association in skeletal muscle following chronic exercise, Eur. J. Appl. Physiol. 104 (2008) 795 802.
[200] M. Bucci, F. Roviezzo, I. Posadas, J. Yu, L. Parente, W.C. Sessa, et al., Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo, Proc. Natl. Acad. Sci. USA 102 (2005) 904 908.
[201] G.I. Lancaster, M.A. Febbraio, Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins, J. Biol. Chem. 280 (2005) 23349 23355.
[202] S. Gupta, A.A. Knowlton, HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway, Am. J. Physiol. Heart Circ. Physiol. 292 (2007) H3052 H3056.
[203] G. Schett, Q. Xu, A. Amberger, R. van der Zee, H. Recheis, J. Willeit, et al., Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity, J. Clin. Invest. 96 (1995) 2569 2577.
[204] G. Schett, B. Metzler, M. Mayr, A. Amberger, D. Niederwieser, R.S. Gupta, et al., Macrophage-lysis mediated by autoantibodies to heat shock protein 65/60, Atherosclerosis 128 (1997) 27 38.

[205] A. Kol, G.K. Sukhova, A.H. Lichtman, P. Libby, Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression, Circulation 98 (1998) 300 307.
[206] A. Kol, A.H. Lichtman, R.W. Finberg, P. Libby, E.A. Kurt-Jones, Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells, J. Immunol. 164 (2000) 13 17.
[207] Q. Xu, H. Dietrich, H.J. Steiner, A.M. Gown, B. Schoel, G. Mikuz, et al., Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock protein 65, Arterioscler. Thromb. 12 (1992) 789 799.
[208] G. Foteinos, A.R. Afzal, K. Mandal, M. Jahangiri, Q. Xu, Anti-heat shock protein 60 autoantibodies induce atherosclerosis in apolipoprotein E-deficient mice via endothelial damage, Circulation 112 (2005) 1206 1213.
[209] M. Dieude, M.A. Gillis, J.F. Theoret, E. Thorin, G. Lajoie, J.S. Levine, et al., Autoantibodies to heat shock protein 60 promote thrombus formation in a murine model of arterial thrombosis, J. Thromb. Haemost. 7 (2009) 710 719.
[210] A.D. Johnson, P.A. Berberian, M. Tytell, M.G. Bond, Atherosclerosis alters the localization of HSP70 in human and macaque aortas, Exp. Mol. Pathol. 58 (1993) 155 168.
[211] M. Tytell, S.G. Greenberg, R.J. Lasek, Heat shock-like protein is transferred from glia to axon, Brain Res. 363 (1986) 161 164.
[212] A.D. Johnson, M. Tytell, Exogenous HSP70 becomes cell associated, but not internalized, by stressed arterial smooth muscle cells, In Vitro Cell. Dev. Biol. Anim. 29A (1993) 807 812.
[213] W. Zhu, P. Roma, A. Pirillo, F. Pellegatta, A.L. Catapano, Human endothelial cells exposed to oxidized LDL express hsp70 only when proliferating, Arterioscler. Thromb. Vasc. Biol. 16 (1996) 1104 1111.
[214] W.F. van, B. Prakken, Heat shock proteins: Darwinistic immune modulation on dangerous grounds, J. Leukoc. Biol. 88 (2010) 431 434.
[215] R.W. Currie, M. Karmazyn, M. Kloc, K. Mailer, Heat-shock response is associated with enhanced postischemic ventricular recovery, Circ. Res. 63 (1988) 543 549.
[216] R.W. Currie, M. Karmazyn, Improved post-ischemic ventricular recovery in the absence of changes in energy metabolism in working rat hearts following heat-shock, J. Mol. Cell. Cardiol. 22 (1990) 631 636.
[217] M. Karmazyn, K. Mailer, R.W. Currie, Acquisition and decay of heat-shock-enhanced postischemic ventricular recovery, Am. J. Physiol. 259 (1990) H424 H431.
[218] M. Okada, N. Hasebe, Y. Aizawa, K. Izawa, J. Kawabe, K. Kikuchi, Thermal treatment attenuates neointimal thickening with enhanced expression of heat-shock protein 72 and suppression of oxidative stress, Circulation 109 (2004) 1763 1768.
[219] C. Brasselet, E. Durand, F. Addad, F. Vitry, G. Chatellier, C. Demerens, et al., Effect of local heating on restenosis and in-stent neointimal hyperplasia in the atherosclerotic rabbit model: a dose-ranging study, Eur. Heart J. 29 (2008) 402 412.
[220] T. Kihara, S. Biro, M. Imamura, S. Yoshifuku, K. Takasaki, Y. Ikeda, et al., Repeated sauna treatment improves vascular endothelial and cardiac function in patients with chronic heart failure, J. Am. Coll. Cardiol. 39 (2002) 754 759.
[221] M. Imamura, S. Biro, T. Kihara, S. Yoshifuku, K. Takasaki, Y. Otsuji, et al., Repeated thermal therapy improves impaired vascular endothelial function in patients with coronary risk factors, J. Am. Coll. Cardiol. 38 (2001) 1083 1088.
[222] J. Jayakumar, K. Suzuki, M. Khan, R.T. Smolenski, A. Farrell, N. Latif, et al., Gene therapy for myocardial protection: transfection of donor hearts with heat shock protein 70 gene protects cardiac function against ischemia-reperfusion injury, Circulation 102 (2000) III302 III306.

[223] J. Jayakumar, K. Suzuki, I.A. Sammut, R.T. Smolenski, M. Khan, N. Latif, et al., Heat shock protein 70 gene transfection protects mitochondrial and ventricular function against ischemia-reperfusion injury, Circulation 104 (2001) I303 I307.
[224] M. Matsumoto, P.C. Dimayuga, C. Wang, J. Kirzner, M. Cercek, J. Yano, et al., Exogenous heat shock protein-70 inhibits cigarette smoke-induced intimal thickening, Am. J. Physiol. Regul. Integr. Comp. Physiol. 295 (2008) R1320 R1327.
[225] T. Anazawa, P.C. Dimayuga, H. Li, S. Tani, J. Bradfield, K.Y. Chyu, et al., Effect of exposure to cigarette smoke on carotid artery intimal thickening: the role of inducible NO synthase, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1652 1658.
[226] S. Tani, P.C. Dimayuga, T. Anazawa, K.Y. Chyu, H. Li, P.K. Shah, et al., Aberrant antibody responses to oxidized LDL and increased intimal thickening in apoE−/− mice exposed to cigarette smoke, Atherosclerosis 175 (2004) 7 14.
[227] E.M. Connolly, C.J. Kelly, G. Chen, T. O'grady, E. Kay, A. Leahy, et al., Pharmacological induction of HSP27 attenuates intimal hyperplasia in vivo, Eur. J. Vasc. Endovasc. Surg. 25 (2003) 40 47.
[228] Y.N. Liu, S.L. Pan, C.Y. Peng, J.H. Guh, D.M. Huang, Y.L. Chang, et al., YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole] inhibits neointima formation in balloon-injured rat carotid through suppression of expressions and activities of matrix metalloproteinases 2 and 9, J. Pharmacol. Exp. Ther. 316 (2006) 35 41.
[229] S. Kummar, M.E. Gutierrez, E.R. Gardner, X. Chen, W.D. Figg, M. Zajac-Kaye, et al., Phase I trial of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), a heat shock protein inhibitor, administered twice weekly in patients with advanced malignancies, Eur. J. Cancer 46 (2010) 340 347.
[230] R.K. Ramanathan, M.J. Egorin, C. Erlichman, S.C. Remick, S.S. Ramalingam, C. Naret, et al., Phase I pharmacokinetic and pharmacodynamic study of 17-dimethylaminoethylamino-17-demethoxygeldanamycin, an inhibitor of heat-shock protein 90, in patients with advanced solid tumors, J. Clin. Oncol. 28 (2010) 1520 1526.
[231] J.E. Lancet, I. Gojo, M. Burton, M. Quinn, S.M. Tighe, K. Kersey, et al., Phase I study of the heat shock protein 90 inhibitor alvespimycin (KOS-1022, 17-DMAG) administered intravenously twice weekly to patients with acute myeloid leukemia, Leukemia 24 (2010) 699 705.
[232] S. Pacey, M. Gore, D. Chao, U. Banerji, J. Larkin, S. Sarker, et al., A Phase II trial of 17-allylamino, 17-demethoxygeldanamycin (17-AAG, tanespimycin) in patients with metastatic melanoma, Invest. New Drugs (2010).
[233] M.A. Biamonte, R. Van de Water, J.W. Arndt, R.H. Scannevin, D. Perret, W.C. Lee, Heat shock protein 90: inhibitors in clinical trials, J. Med. Chem. 53 (2010) 3 17.
[234] T. Uchiyama, H. Atsuta, T. Utsugi, Y. Ohyama, T. Nakamura, A. Nakai, et al., Simvastatin induces heat shock factor 1 in vascular endothelial cells, Atherosclerosis 188 (2006) 265 273.
[235] G. Niu, X. Chen, From protein-protein interaction to therapy response: molecular imaging of heat shock proteins, Eur. J. Radiol. 70 (2009) 294 304.
[236] M. Benagiano, M.M. D'Elios, A. Amedei, A. Azzurri, R. van der Zee, A. Ciervo, et al., Human 60-kDa heat shock protein is a target autoantigen of T cells derived from atherosclerotic plaques, J. Immunol. 174 (2005) 6509 6517.
[237] Q. Xu, Role of heat shock proteins in atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1547 1559.
[238] D. Harats, N. Yacov, B. Gilburd, Y. Shoenfeld, J. George, Oral tolerance with heat shock protein 65 attenuates Mycobacterium tuberculosis-induced and high-fat-diet-driven atherosclerotic lesions, J. Am. Coll. Cardiol. 40 (2002) 1333 1338.

[239] S.A. Huber, P. Sakkinen, C. David, M.K. Newell, R.P. Tracy, T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia, Circulation 103 (2001) 2610 2616.
[240] R. Maron, G. Sukhova, A.M. Faria, E. Hoffmann, F. Mach, P. Libby, et al., Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice, Circulation 106 (2002) 1708 1715.
[241] G.H. van Puijvelde, E.T. van, E.J. van Wanrooij, K.L. Habets, V.P. de, R. van der Zee, Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 2677 2683.
[242] Q. Xiong, J. Li, L. Jin, J. Liu, T. Li, Nasal immunization with heat shock protein 65 attenuates atherosclerosis and reduces serum lipids in cholesterol-fed wild-type rabbits probably through different mechanisms, Immunol. Lett. 125 (2009) 40 45.
[243] E.W. Van, G. Wick, S. Albani, I. Cohen, Stress, heat shock proteins, and autoimmunity: how immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases, Ann. NY Acad. Sci. 1113 (2007) 217 237.

CELL STRESS PROTEINS IN ATHEROTHROMBOSIS

Julio Madrigal-Matute, Roxana Martinez-Pinna, Carlos Ernesto Fernandez-García, Priscila Ramos-Mozo, Elena Burillo, Jesus Egido, Luis Miguel Blanco-Colio, Jose Luis Martin-Ventura.

Vascular Research Laboratory, IIS-Fundacion Jimenez Diaz, Autonoma University, Madrid, Spain.

Number of words (body) = 3360 (without references)/ 7444 (with references)

Corresponding author:

Julio Madrigal Matute

Vascular Research Lab.

IIS-Fundacion Jimenez Diaz

Autonoma University

Av. Reyes Católicos 2

28040. Madrid. Spain

Phone: +34915504800

Fax: +34915504399

jmadrigal@fjd.es

Abstract

Cell stress proteins (CSPs) are a large and heterogenous family of proteins, sharing two main characteristics; their levels and/or location are modified under stress and most of them can exert a chaperon function inside the cells. Nonetheless, they are also involved in the modulation of several mechanisms, both at the intracellular and the extracellular compartments. There are more than 100 proteins belonging to the CSPs family, among them the Thioredoxin (TRX) system, which is the focus of the present review. TRX system is composed of several proteins such as TRX and Peroxiredoxin (PRDX), two thiol containing enzymes which are key players in redox homeostasis due to their ability to scavenge potential harmful reactive oxygen species. In addition to their main role as antioxidants, recent data highlights their function in several processes such as cell signalling, immune inflammatory responses or apoptosis, all of them key mechanisms involved in atherothrombosis. Moreover, since TRX and PRDX are present in the pathological vascular wall and can be secreted under pro-oxidative conditions to the circulation, several studies have addressed their role as diagnostic, prognostic and therapeutic biomarkers of cardiovascular diseases (CVD).

1. Introduction

The vast majority of the proteins require further assistance for acquiring proper maturation and stability, this process described in the late 80s is facilitated through the activity of a family of proteins called "molecular chaperones" or cell stress proteins (CSPs)[1]. This large and diverse group of proteins composed by more than 20 families of proteins and more than 100 proteins includes the Heat shock proteins (HSPs) and the Thioredoxin (TRX) system. The role of HSPs in cardiovascular diseases (CVDs) has been thoroughly reviewed [2]. In the present review, we will focus on the role of the TRX system, specifically TRX and Peroxiredoxins (PRDXs) on atherothrombosis.

2. The Thioredoxin system

The TRX system mainly comprises TRX, TRX reductase, TRX interacting protein [(TXNIP), vitamin D3–upregulated protein-1 ((VDUP)-1) or TRX-binding protein (TRXBP)] and the PRDXs [3]. The TRX system is involved in protein assembly and exerts a key role in cellular redox maintenance.

Under physiological conditions, intra and extracellular reactive oxygen species (ROS) modulate metabolic, signaling and transcriptional processes within the cell.

However, pathological dysregulation of the redox balance could contribute to CVDs [4, 5]. Cellular redox homeostasis is tightly regulated by the coordinated action of NADPH oxidases, the TRX system and glutathione (GSH) [6], among others. The TRX system and GSH are thiol reduction systems with a key role in the defense against excessive ROS production, as well as in the modulation of signaling processes such as inflammation, cellular proliferation and apoptosis [7-9]. These molecules maintain the intracellular milieu in a reduced state. GSH is used by the GSH peroxidase to reduce peroxides, producing oxidized GSH (GSSG) while GSH reductase reduces this oxidized form to GSH. The antioxidant properties of TRX result from PRDX action, which recycle H_2O_2 through reduction of several hydroperoxides into water and alcohol (Figure 1).

Depending on their cellular location, TRX/PRDX may exert different functions than their known chaperone and antioxidant activities. This process can be described with the so called "Moonlighting proteins" theory [10]. This idea supports the notion that one gene = one protein = one function is simple and old-fashioned because large number of proteins have two or more functions. This theory might not apply to every protein but it seems to be right for TRX/PRDX. Under certain circumstances, mainly pro-oxidative conditions, TRX and PRDX could be released to the extracellular milieu [11, 12], although their trafficking mechanisms are not yet fully described.

2.1. Intracellular TRX

TRXs are a class of small redox molecules (~12 kDa) present in prokaryotes and eukaryotes and are essential for cellular viability [13]. So far, 3 human TRX variants have been characterized codified by different genes. TRX1 is localized under resting conditions in the cytosol but can be translocated upon stress conditions[14] [e.g. it can be found in an oxidized form in the nucleus of exponential growing cells[15], (Figure 2)]; TRX2 is mitochondrial [16, 17]; and SpTRX [18] is abundantly expressed in spermatozoa. TRX has a redox active disulfide/dithiol site within 2 conserved cys residues [19], and it functions as an antioxidant molecule by protecting cells against H_2O_2 [20], regulating Heme-oxygenase 1 (HO-1) expression [21] or inducing manganese superoxide dismutase (MnSOD) in the mitochondria [22]. Moreover, it has a protective role against nitric oxide (NO) induced stress, regulating NO synthases activity [23] and through other NO regulating processes [24]. In addition to its role as antioxidant protein, TRX interacts with numerous signaling molecules [including Apoptosis signal-regulating kinase 1 (ASK1) and TXNIP] and transcription factors such as Nuclear factor-kappa B (NF-κB) and Activating protein 1 (AP-1). TRX function can be regulated by redox modification (e.g

NO increases S-nitrosylation of TRX stimulating TRX activity [25]) or by TXNIP binding [26] (reducing TRX activity), among other mechanisms.

Regarding intracellular TRX *in vivo* functions, as TRX-1 KO mice are lethal, some information has been obtained from studies performed in TRX transgenic (Tg) mice [27]. TRX overexpression in mice protects placenta from oxidative stress and fetal growth by augmenting glucose availability [28] and it also functions as a shelter against apoptosis induced by extensive oxidation in diabetic embryophaty [29] or streptozotocin-induced diabetic osteopenia [30]. Furthermore, TRX is able to protect the lung injury provoked by diesel exhaust particles (DEP) derived from diesel engine-powered automobiles and industrial machines. This protective role, exerted through AKT modulation, is reflected in the augmented TRX levels in control cells induced by DEP [31]. Also, mediated by AKT signaling, TRX protects neurons against apoptosis during brain focal ischemia [32].

Thus, the major role of TRX in the redox balance is supported by the numerous data regarding the protection exerted by TRX against excessive oxidative damage in different diseases and the embryonic lethality of KO mice for TRX.

2.2. Intracellular PRDX

PRDXs are a recent discovery among the peroxidases lacking the hemo group. PRDX protein levels are very abundant, around 0.1-1 % of total soluble protein in mammals, and they are ubiquitously distributed in all organisms [12, 33]. PRDXs are thiol specific enzymes lacking selenium and they use their active sites to reduce peroxides and scavenge ROS [12]. Mammal cells express 6 PRDX isoforms (PRDX 1-6); 1, 2, and 6 are cytosolic, 3 and 5 are mitochondrial, while 4 is described as a secretory protein in most tissues [34]. Every isoform contains a conserved Cys residue, which is the primary site for H_2O_2 oxidation, and they can be further classified according to their Cys residues (PRDX1-4 belong to the 2-Cys subfamily, PRDX5 or atypical 2-Cys and PRDX6 or 1-Cys PRDX) [35].

PRDXs scavenge H_2O_2 more efficiently than other systems such as catalase due to its higher affinity for H_2O_2 [36]. PRDX can modulate NADPH oxidase activity through H_2O_2 inactivation [37]. In addition, PRDX can also reduce peroxynitrites levels through peroxynitrites reductases [38]. Among the mechanisms modifying PRDX functions, several posttranslational modifications have been described, such as nitrosylation [39], glutathionylation [40], phosphorylation and the hyperoxidation of its active site [41] which stimulates its chaperone activity [42]. For example, under low H_2O_2 concentrations

produced in conditions of cellular homeostasis PRDX forms low molecular weight oligomers, exerting peroxidase activity. However, under significant changes in H_2O_2 concentration, PRDX experiences structural changes and forms high molecular weight oligomers and acquires chaperone activity [43, 44]. Similarly to TRX, PRDX interacts with several proteins (e.g. cyclophilin A, macrophage inhibitory factor, etc) and can modulate the function of these binding proteins, in a dependent or independent manner of the PRX redox status [35].

Mice lacking PRDX1 are viable with a phenotype characterized by hemolytic anemia caused by an increased ROS production by red blood cells (RBCs). Furthermore, PRDX1 has tumor suppressor properties as PRDX1 KO mice shows an increased rate in malignant tumors as they age, which can be explained by the excessive accumulation of damaged tissue due to extreme ROS production [45]. Similarly to PRDX1 KO mice, targeted disruption of PRDX2 causes cysteine oxidation of several proteins on RBCs membranes, which finally results in augmented levels of denatured protein, cell toxification and hemolysis [46]. PRDX2 also has been identified as a tumor suppressor gene [47]. Accordingly, PRDX3 (also known as MER5) KO mice are characterized by increased ROS production in macrophages and develope more severe lung injury upon Lypopolisaccharyde (LPS) induction, possibly due to an excessive DNA and protein oxidative damage and inflammatory cell infiltration [48]. In fact, it has been calculated that almost 90% of H_2O_2 targets PRDX3 within the mitochondrial matrix, playing a major role in redox signaling in the mitochondria [49], protecting the cells against apoptosis induced by excessive damage to mitochondrial macromolecules [50]. PRDX3 absence is also involved in mitochondrial dysregulation associated to obesity, through increased protein carbonylation and ROS production [51]. It is noteworthy that PRDX3 KO adipocytes accumulate more fat than wild type due to hypertrophy and defects in the levels of enzymes implicated in glucose/lipid metabolism [51]. PRDX4 is mostly a secretory protein, while it is attached to the endoplasmic reticulum (ER) membrane of spermatogenic cells in mature testes. In these cells PRDX4 protects from cell death through its anti-oxidant properties, nonetheless PRDX4 KO spermatozoa shows normal fertilization [52]. To our knowledge there is no PRDX5 KO strain, although it has been described that PRDX5 overexpression prevents ROS production and p53 dependent apoptosis [53]. Nevertheless, KO mice for PRDX6, the only 1-Cys member of the peroxiredoxin family, were more vulnerable to ischemic reperfusion injury as shown by increased infarct size and higher amount oxidative stress

[54]. In agreement, PRDX6 overexpression functions as a shelter in mouse lungs against toxicity of hyperoxia [55].

On the whole, the plethora of data regarding the different isoforms of PRDX demonstrate that every subunit, independently of its location, are members of one of the major cellular systems in charge of scavenging pro-oxidant species and thus in the maintenance of cellular redox status.

2.3. Extracellular TRX

TRX expression can be augmented very fast under stress and is secreted by normal and tumor cells although its secretion does not seem to follow a classical Golgi apparatus pathway [11]. TRX location is regulated by TXNIP binding [26] and facilitates TRX transport from cytoplasm to the membrane under oxidative stress [14]. Another mechanism that can be involved in TRX active secretion is the exosomal pathway. Exosomes are small microparticles released by cells upon activation or apoptosis. These vesicles have been implicated in thrombosis, diabetes, inflammation, atherosclerosis and vascular cell proliferation [56]. Proteomic studies have described the presence of TRX in exosomes in B cells [57], bladder cancer cells [58], colorectal cancer cells [59] and urine [60] (Figure 2).

There is also a truncated form of TRX which corresponds to the last 80-84 aminoacids from the N-terminal end, named TRX80, and it is present in plasma where it was firstly identified as a stimulating factor of eosinophils cytotoxicity [61]. It is possibly a result of protease activity but this proccess is still unknown (Figure 2). Recombinant TRX80 is a potent mitogenic cytokine for Peripheral blood mononuclear cells (PBMCs), an effect not shared by TRX [62]. TRX80 differs from TRX because it forms a dimer lacking reductase activity and its activity is independent of the Cys residues from the TRX active site. The main cellular target for TRX80 are PBMCs in which it induces a Th1 response via IL12 production [63].

Extracellular TRX is present in the circulation and its levels are increased under oxidative stress or inflammation [64] (Figure 2). TRX has been pointed out as a biomarker in numerous oxidative and inflammatory diseases such as rheumatoid arthritis (RA) in which plasma TRX levels of normal subjects were significantly lower than those of RA patients and correlated with RA disease activity and C-reactive protein [65]. TRX levels were increased in patients with systemic inflammatory stress syndrome (SIRS)/sepsis compared to control subjects [66].

2.4. Extracellular PRDX

PRDX1 can be found inside the Golgi apparatus on endothelial cells (ECs) [67], and under Phorbol 12-myristate 13-acetate (PMA) stimulation PRDX1 is translocated to the cellular membrane [68], as also showed for PRDX6 in polymorphonuclear cells (PMNs) [69] (Figure 2). PRDX might also be secreted by lung cancer cells through a non-classical pathway [70, 71]. Nonetheless, the extracellular function of PRDX is still unknown. Unlike the well described function of intracellular PRDX1, membrane PRDX6 helps in the maintenance of an optimal NADPH oxidase activity [69]. A number of chaperones, including TRX and HSPs are released by stressed or dying cells, acting as an endogenous warning system through binding of these signals to receptors on the outer membrane [72-75]. Most of these signals are recognized by Toll like receptor 4 (TLR4) [74, 75]. Accordingly, PRDX1 binds TLR4 and stimulates pro-inflammatory cytokine production in macrophages and dendritic cells, which suggests that PRDX could be acting as damage associated molecular-pattern molecule (DAMP). Its trafficking seems to be dependent on PRDX binding to *Protein kinase C* (PKC) through microvesicles [76]. In fact, exosomes can be participating in active transport of PRDX since proteomic studies have described PRDX in exosomes in B cells [57], bladder cancer cells [58], breast cancer cells [77], breast milk [78], colorectal cancer cells [59] and saliva [79] (Figure 2).

Thus, it is tempting to speculate that extracellular levels of PRDX/TRX result from a cellular response to high oxidant conditions in the outer milieu.

3. TRX/PRDX in atherothrombosis

Atherothrombosis is an immune-inflammatory disease, originated by the subendothelial acummulation of LDLs, that can be oxydised by ROS. Oxidative stress is not only involved in the first stages of atherogenesis by modifying LDLs or NO, but also in later stages of atherothrombosis by modulating the expression of proteases that weakens the fibrous cap [80, 81]. ROS overproduction also produce direct damage to macromolecules such as lipids, nucleic acids and proteins [82]. Furthermore, ROS can act as signaling molecules by inducing the activation of several cells from the vasculature. For example, through LDL oxydation and/or direct cell targeting, ROS can induce endothelial dysfunction and further leucocyte activation, deposition and extravasation. In adittion, ROS are clearly involved in the activation of vascular smooth muscle cells (VSMCs) from the lesion and sustain foam cell formation. Thus, pathological ROS

overproduction is a main feature in atherogenesis and plaque rupture, which finally results in almost 70% of the clinical events [83].

Among the different systems involved in the redox maintenance in the vasculature, one of the most active is the TRX system. It is present in ECs [14, 84], VSMCs [85, 86], monocytes/macrophages [87, 88], RBCs [89, 90] and PMNs [91, 90]. Since TRX and PRDX are present in the atherosclerotic plaque and they can modulate different mechanisms involved in CVDs, several studies have addressed their role as diagnostic, prognostic and therapeutic biomarkers.

3.1. TRX

TRX is abundantly expressed in the vasculature and its levels are increased under oxidative stress, possibly as a response mechanism to augmented ROS production [19]. Besides, TRX expression in the endothelium and in macrophages is augmented in atherosclerotic patients [92] and in arteries damaged by the balloon model [93]. More recently, TRX has been pointed as a possible marker for unstable atherosclerotic plaques due to its association with oxidative stress and intraplaque hemorrhage in culprit lesions [94]. TRX reductase is as well overexpressed in atherosclerotic plaques, maybe synthesized by macrophages engulfing oxLDLs [95].

The antioxidant effects of TRX are shown when overexpressed in mouse hearts, protecting them from myocardial infarction and displaying significative improved post-ischemic ventricular recovery [96]. The positive effects of TRX in myocardial infarction are also due to its neoangiogenic properties as shown in different murine models [97, 98].

On the other hand, TRX can function as a signaling molecule by decreasing pressure-overload cardiac hypertrophy [99], maybe through upregulation of miR-98 [100]. Although, there is a controversy about this matter because almost at the same time it was published another article in which the authors described that activation of TRX participates in the development of pressure-overload cardiac hypertrophy. In this respect, animals overexpressing TXNIP developed less hypertrophy [101]. Furthermore, transverse aortic constriction increased TRX activity accompanied by a 40 % reduction in TXNIP levels [101]. If variations in TXNIP and TRX levels/activity reflect an increase oxidative stress or they act as signaling molecules is still a matter to elucidate [102].

Recent studies have shown that extracellular TRX can inhibit Interleukin-1 expression stimulated by LPS in monocyte-derived macrophages [103]. Besides, TRX1 administration has beneficial effects on myosin-induced autoimmune myocarditis through inhibition of inflammatory mediators and macrophage infiltration [104] and TRX1

also protects from against reperfusion-induced arrhythmias [105]. Furthermore, TRX administration has been also shown to be beneficial in cerebral ischemia/reperfusion injury reducing the infarcted area through its anti-oxidant properties [106]. As an additional support for the beneficial effects of TRX in therapeutics, it is to note that TRX-1 gene delivery protects hypertensive rats from myocardial infarction through HO-1/B-cell lymphoma 2 (BCL-2) [107].

Regarding cardiovascular diseases, TRX levels are elevated in plasma from atherothrombotic patients [108, 109], and high homocysteine plus low TRX is related to CAD severity [110]. Furthermore, TRX was elevated in patients following angioplasty and there was an association with increased TRX levels and decreased rate of restenosis at follow-up angiography (6 months) [111]. We have recently reported an increase in serum TRX from Abdominal aortic aneurysm (AAA) patients compared with control subjects. Besides, TRX correlates with AAA size and expansion rate which suggests that TRX could be a good biomarker of AAA evolution [91].

3.2. PRDX

PRDX expression can be modified by pro-oxidative stimulus such as LPS or the phorbol ester 12-O-tetradecanoylphorbol- 13-acetate (TPA) [88, 112]. Attention to PRDX as a major regulator of H_2O_2 homeostasis is growing [34]. In cells stimulated with platelet-derived growth factor (PDGF) or tumor necrosis factor alpha, PRDX overexpression or silencing provoked respectively reduction or increase of H_2O_2 levels [113]. Moreover, through H_2O_2 scavenging, PRDX can inhibit the NF-κB pathway and consequently the inflammatory response [114]. Different PRDX isoforms seem to modulate different cellular responses. For example, transfection of VSMCs from rat pulmonary artery with an expression plasmid for PRDX1, increases proliferation rate significatively [115]. PRDX1 also diminishes leucocytes activation and adhesion to vascular endothelium. Double KO mice for PRDX1 and ApoE chow fed showed larger atherosclerotic lesions and macrophages enriched than ApoE KO mice [116]. Mice KO for PRDX2/ApoE showed exacerbated atherosclerotic lesion formation dependent both in vascular and hematopoietic cells. Besides, immune cells accumulation in the atherosclerotic lesions is increased due to PRDX2 absence and also redox dependent signaling PRDX2 [117]. Moreover, PRDX2 modulates PDGF signaling, inhibiting thereby cell proliferation and migration [113, 118]. Using different *in vivo* models it was shown that CD36 KO mice produce lower levels of ROS, along with and increased in Heme-oxygenase1 (HO-1) and PRDX2. Furthermore, NF-E2–related factor-2 (Nrf2), a transcription factor in

charge of the transcriptional regulation of HO-1 and PRDX2, knockdown leaded to increased ROS production and a pro-thrombotic phenotype under $FeCl_3$ treatment, a similar phenotype to that of PRDX2 KO mice [119].

Besides, PRDX3 overexpression prevents ventricular remodeling and cardiac failure post-myocardial infarction in mice [120]. As mention above, PRDX6 protects mice against ischemic reperfusion injury [54]. Although, little is known about circulating levels of PRDX, we have recently described high PRDX1 levels in serum from AAA patients [90]. Besides, PRDX1 levels correlated positively with size and expansion rate of AAA, suggesting its potential role as diagnostic and prognostic biomarker for AAA.

4. Conclusion

On the whole, we have summarized several findings that demonstrate the major role of the TRX system in the maintenance of the redox status in CVDs. Furthermore, the extracellular levels of PRDX/TRX seem to be related with a pro-oxidative scenario and there is growing data suggesting their potential role as biomarkers for oxidative-related diseases. Finally, their value as useful therapeutic tools is being tested and future studies are necessary to validate its prospective beneficial effects in CVDs.

FUNDING

This work was supported by the Spanish Ministerio de Ciencia y Tecnología (SAF 2010-21852), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (PI10/00072) and Sociedad Española de Aterosclerosis and Lilly.

REFERENCES

(1) Hemmingsen SM, Woolford C, van der Vies SM, Tilly K, Dennis DT, Georgopoulos CP, Hendrix RW, Ellis RJ. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* 1988 May 26;333(6171):330-4.

(2) Madrigal-Matute J, Martin-Ventura JL, Blanco-Colio LM, Egido J, Michel JB, Meilhac O. Heat-shock proteins in cardiovascular disease. *Adv Clin Chem* 2011;54:1-43.

(3) Powis G, Briehl M, Oblong J. Redox signalling and the control of cell growth and death. *Pharmacol Ther* 1995;68(1):149-73.

(4) Albertini R, Moratti R, De LG. Oxidation of low-density lipoprotein in atherosclerosis from basic biochemistry to clinical studies. *Curr Mol Med* 2002 September;2(6):579-92.

(5) Lavrovsky Y, Chatterjee B, Clark RA, Roy AK. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp Gerontol* 2000 August;35(5):521-32.

(6) World CJ, Yamawaki H, Berk BC. Thioredoxin in the cardiovascular system. *J Mol Med* 2006 December;84(12):997-1003.

(7) Holmgren A. Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxid Redox Signal* 2000;2(4):811-20.

(8) Powis G, Montfort WR. Properties and biological activities of thioredoxins. *Annu Rev Biophys Biomol Struct* 2001;30:421-55.

(9) Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 2003 April 25;300(5619):650-3.

(10) Jeffery CJ. Moonlighting proteins. *Trends Biochem Sci* 1999 January;24(1):8-11.

(11) Rubartelli A, Bajetto A, Allavena G, Wollman E, Sitia R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J Biol Chem* 1992 December 5;267(34):24161-4.

(12) Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 2005 June 15;38(12):1543-52.

(13) Slaby I, Holmgren A. Thioredoxin reductase-dependent insulin disulfide reduction by phage T7 DNA polymerase reflects dissociation of the enzyme into subunits. *J Biol Chem* 1989 October 5;264(28):16502-6.

(14) World C, Spindel ON, Berk BC. Thioredoxin-interacting protein mediates TRX1 translocation to the plasma membrane in response to tumor necrosis factor-alpha: a key mechanism for vascular endothelial growth factor receptor-2 transactivation by reactive oxygen species. *Arterioscler Thromb Vasc Biol* 2011 August;31(8):1890-7.

(15) Spielberger JC, Moody AD, Watson WH. Oxidation and nuclear localization of thioredoxin-1 in sparse cell cultures. *J Cell Biochem* 2008 August 1;104(5):1879-89.

(16) Spyrou G, Enmark E, Miranda-Vizuete A, Gustafsson J. Cloning and expression of a novel mammalian thioredoxin. *J Biol Chem* 1997 January 31;272(5):2936-41.

(17) Tanaka T, Hosoi F, Yamaguchi-Iwai Y, Nakamura H, Masutani H, Ueda S, Nishiyama A, Takeda S, Wada H, Spyrou G, Yodoi J. Thioredoxin-2 (TRX-2) is an

essential gene regulating mitochondria-dependent apoptosis. *EMBO J* 2002 April 2;21(7):1695-703.

(18) Miranda-Vizuete A, Ljung J, Damdimopoulos AE, Gustafsson JA, Oko R, Pelto-Huikko M, Spyrou G. Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. *J Biol Chem* 2001 August 24;276(34):31567-74.

(19) Nakamura H, Nakamura K, Yodoi J. Redox regulation of cellular activation. *Annu Rev Immunol* 1997;15:351-69.

(20) Nakamura H, Matsuda M, Furuke K, Kitaoka Y, Iwata S, Toda K, Inamoto T, Yamaoka Y, Ozawa K, Yodoi J. Adult T cell leukemia-derived factor/human thioredoxin protects endothelial F-2 cell injury caused by activated neutrophils or hydrogen peroxide. *Immunol Lett* 1994 September;42(1-2):75-80.

(21) Trigona WL, Mullarky IK, Cao Y, Sordillo LM. Thioredoxin reductase regulates the induction of haem oxygenase-1 expression in aortic endothelial cells. *Biochem J* 2006 February 15;394(Pt 1):207-16.

(22) Das KC, Lewis-Molock Y, White CW. Elevation of manganese superoxide dismutase gene expression by thioredoxin. *Am J Respir Cell Mol Biol* 1997 December;17(6):713-26.

(23) Zhang J, Li YD, Patel JM, Block ER. Thioredoxin overexpression prevents NO-induced reduction of NO synthase activity in lung endothelial cells. *Am J Physiol* 1998 August;275(2 Pt 1):L288-L293.

(24) Shao LE, Tanaka T, Gribi R, Yu J. Thioredoxin-related regulation of NO/NOS activities. *Ann N Y Acad Sci* 2002 May;962:140-50.

(25) Haendeler J, Hoffmann J, Zeiher AM, Dimmeler S. Antioxidant effects of statins via S-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. *Circulation* 2004 August 17;110(7):856-61.

(26) Junn E, Han SH, Im JY, Yang Y, Cho EW, Um HD, Kim DK, Lee KW, Han PL, Rhee SG, Choi I. Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol* 2000 June 15;164(12):6287-95.

(27) Matsui M, Oshima M, Oshima H, Takaku K, Maruyama T, Yodoi J, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol* 1996 August 25;178(1):179-85.

(28) Umekawa T, Sugiyama T, Kihira T, Murabayashi N, Zhang L, Nagao K, Kamimoto Y, Ma N, Yodoi J, Sagawa N. Overexpression of thioredoxin-1 reduces

oxidative stress in the placenta of transgenic mice and promotes fetal growth via glucose metabolism. *Endocrinology* 2008 August;149(8):3980-8.

(29) Kamimoto Y, Sugiyama T, Kihira T, Zhang L, Murabayashi N, Umekawa T, Nagao K, Ma N, Toyoda N, Yodoi J, Sagawa N. Transgenic mice overproducing human thioredoxin-1, an antioxidative and anti-apoptotic protein, prevents diabetic embryopathy. *Diabetologia* 2010 September;53(9):2046-55.

(30) Hamada Y, Fujii H, Kitazawa R, Yodoi J, Kitazawa S, Fukagawa M. Thioredoxin-1 overexpression in transgenic mice attenuates streptozotocin-induced diabetic osteopenia: a novel role of oxidative stress and therapeutic implications. *Bone* 2009 May;44(5):936-41.

(31) Kaimul AM, Nakamura H, Tanito M, Yamada K, Utsumi H, Yodoi J. Thioredoxin-1 suppresses lung injury and apoptosis induced by diesel exhaust particles (DEP) by scavenging reactive oxygen species and by inhibiting DEP-induced downregulation of Akt. *Free Radic Biol Med* 2005 December 15;39(12):1549-59.

(32) Zhou F, Gomi M, Fujimoto M, Hayase M, Marumo T, Masutani H, Yodoi J, Hashimoto N, Nozaki K, Takagi Y. Attenuation of neuronal degeneration in thioredoxin-1 overexpressing mice after mild focal ischemia. *Brain Res* 2009 May 26;1272:62-70.

(33) Wood ZA, Schroder E, Robin HJ, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 2003 January;28(1):32-40.

(34) Chae HZ, Kim HJ, Kang SW, Rhee SG. Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Res Clin Pract* 1999 September;45(2-3):101-12.

(35) Rhee SG, Woo HA. Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger HO, and protein chaperones. *Antioxid Redox Signal* 2011 August 1;15(3):781-94.

(36) Peskin AV, Low FM, Paton LN, Maghzal GJ, Hampton MB, Winterbourn CC. The high reactivity of peroxiredoxin 2 with H(2)O(2) is not reflected in its reaction with other oxidants and thiol reagents. *J Biol Chem* 2007 April 20;282(16):11885-92.

(37) Leavey PJ, Gonzalez-Aller C, Thurman G, Kleinberg M, Rinckel L, Ambruso DW, Freeman S, Kuypers FA, Ambruso DR. A 29-kDa protein associated with p67phox expresses both peroxiredoxin and phospholipase A2 activity and enhances superoxide anion production by a cell-free system of NADPH oxidase activity. *J Biol Chem* 2002 November 22;277(47):45181-7.

(38) Uwayama J, Hirayama A, Yanagawa T, Warabi E, Sugimoto R, Itoh K, Yamamoto M, Yoshida H, Koyama A, Ishii T. Tissue Prx I in the protection against Fe-NTA and the reduction of nitroxyl radicals. *Biochem Biophys Res Commun* 2006 January 6;339(1):226-31.

(39) Fang J, Nakamura T, Cho DH, Gu Z, Lipton SA. S-nitrosylation of peroxiredoxin 2 promotes oxidative stress-induced neuronal cell death in Parkinson's disease. *Proc Natl Acad Sci U S A* 2007 November 20;104(47):18742-7.

(40) Chae HZ, Oubrahim H, Park JW, Rhee SG, Chock PB. Protein glutathionylation in the regulation of peroxiredoxins: a family of thiol-specific peroxidases that function as antioxidants, molecular chaperones, and signal modulators. *Antioxid Redox Signal* 2012 March 15;16(6):506-23.

(41) Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K, Rhee SG. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *J Biol Chem* 2002 October 11;277(41):38029-36.

(42) Barranco-Medina S, Lazaro JJ, Dietz KJ. The oligomeric conformation of peroxiredoxins links redox state to function. *FEBS Lett* 2009 June 18;583(12):1809-16.

(43) Lim JC, Choi HI, Park YS, Nam HW, Woo HA, Kwon KS, Kim YS, Rhee SG, Kim K, Chae HZ. Irreversible oxidation of the active-site cysteine of peroxiredoxin to cysteine sulfonic acid for enhanced molecular chaperone activity. *J Biol Chem* 2008 October 24;283(43):28873-80.

(44) Rhee SG, Jeong W, Chang TS, Woo HA. Sulfiredoxin, the cysteine sulfinic acid reductase specific to 2-Cys peroxiredoxin: its discovery, mechanism of action, and biological significance. *Kidney Int Suppl* 2007 August;(106):S3-S8.

(45) Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. *Nature* 2003 July 31;424(6948):561-5.

(46) Lee TH, Kim SU, Yu SL, Kim SH, Park DS, Moon HB, Dho SH, Kwon KS, Kwon HJ, Han YH, Jeong S, Kang SW, Shin HS, Lee KK, Rhee SG, Yu DY. Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. *Blood* 2003 June 15;101(12):5033-8.

(47) Agrawal-Singh S, Isken F, Agelopoulos K, Klein HU, Thoennissen NH, Koehler G, Hascher A, Baumer N, Berdel WE, Thiede C, Ehninger G, Becker A, Schlenke P,

Wang Y, McClelland M, Krug U, Koschmieder S, Buchner T, Yu DY, Singh SV, Hansen K, Serve H, Dugas M, Muller-Tidow C. Genome wide analysis of histone H3 acetylation patterns in AML identifies PRDX2 as an epigenetically silenced tumor suppressor gene. *Blood* 2011 December 29.

(48) Li L, Shoji W, Takano H, Nishimura N, Aoki Y, Takahashi R, Goto S, Kaifu T, Takai T, Obinata M. Increased susceptibility of MER5 (peroxiredoxin III) knockout mice to LPS-induced oxidative stress. *Biochem Biophys Res Commun* 2007 April 13;355(3):715-21.

(49) Cox AG, Winterbourn CC, Hampton MB. Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. *Biochem J* 2010 January 15;425(2):313-25.

(50) Chang TS, Cho CS, Park S, Yu S, Kang SW, Rhee SG. Peroxiredoxin III, a mitochondrion-specific peroxidase, regulates apoptotic signaling by mitochondria. *J Biol Chem* 2004 October 1;279(40):41975-84.

(51) Huh JY, Kim Y, Jeong J, Park J, Kim I, Huh KH, Kim YS, Woo HA, Rhee SG, Lee KJ, Ha H. Peroxiredoxin 3 is a key molecule regulating adipocyte oxidative stress, mitochondrial biogenesis, and adipokine expression. *Antioxid Redox Signal* 2012 February 1;16(3):229-43.

(52) Iuchi Y, Okada F, Tsunoda S, Kibe N, Shirasawa N, Ikawa M, Okabe M, Ikeda Y, Fujii J. Peroxiredoxin 4 knockout results in elevated spermatogenic cell death via oxidative stress. *Biochem J* 2009 April 1;419(1):149-58.

(53) Zhou Y, Kok KH, Chun AC, Wong CM, Wu HW, Lin MC, Fung PC, Kung H, Jin DY. Mouse peroxiredoxin V is a thioredoxin peroxidase that inhibits p53-induced apoptosis. *Biochem Biophys Res Commun* 2000 February 24;268(3):921-7.

(54) Nagy N, Malik G, Fisher AB, Das DK. Targeted disruption of peroxiredoxin 6 gene renders the heart vulnerable to ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2006 December;291(6):H2636-H2640.

(55) Manevich Y, Fisher AB. Peroxiredoxin 6, a 1-Cys peroxiredoxin, functions in antioxidant defense and lung phospholipid metabolism. *Free Radic Biol Med* 2005 June 1;38(11):1422-32.

(56) Azevedo LC, Pedro MA, Laurindo FR. Circulating microparticles as therapeutic targets in cardiovascular diseases. *Recent Pat Cardiovasc Drug Discov* 2007 January;2(1):41-51.

(57) Buschow SI, van Balkom BW, Aalberts M, Heck AJ, Wauben M, Stoorvogel W. MHC class II-associated proteins in B-cell exosomes and potential functional implications for exosome biogenesis. *Immunol Cell Biol* 2010 November;88(8):851-6.

(58) Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, Mason MD, Clayton A. Proteomics analysis of bladder cancer exosomes. *Mol Cell Proteomics* 2010 June;9(6):1324-38.

(59) Choi DS, Lee JM, Park GW, Lim HW, Bang JY, Kim YK, Kwon KH, Kwon HJ, Kim KP, Gho YS. Proteomic analysis of microvesicles derived from human colorectal cancer cells. *J Proteome Res* 2007 December;6(12):4646-55.

(60) Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, Wang NS, Knepper MA. Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol* 2009 February;20(2):363-79.

(61) Dessein AJ, Lenzi HL, Bina JC, Carvalho EM, Weiser WY, Andrade ZA, David JR. Modulation of eosinophil cytotoxicity by blood mononuclear cells from healthy subjects and patients with chronic schistosomiasis mansoni. *Cell Immunol* 1984 April 15;85(1):100-13.

(62) Pekkari K, Gurunath R, Arner ES, Holmgren A. Truncated thioredoxin is a mitogenic cytokine for resting human peripheral blood mononuclear cells and is present in human plasma. *J Biol Chem* 2000 December 1;275(48):37474-80.

(63) Pekkari K, Avila-Carino J, Bengtsson A, Gurunath R, Scheynius A, Holmgren A. Truncated thioredoxin (Trx80) induces production of interleukin-12 and enhances CD14 expression in human monocytes. *Blood* 2001 May 15;97(10):3184-90.

(64) Kondo N, Ishii Y, Kwon YW, Tanito M, Horita H, Nishinaka Y, Nakamura H, Yodoi J. Redox-sensing release of human thioredoxin from T lymphocytes with negative feedback loops. *J Immunol* 2004 January 1;172(1):442-8.

(65) Jikimoto T, Nishikubo Y, Koshiba M, Kanagawa S, Morinobu S, Morinobu A, Saura R, Mizuno K, Kondo S, Toyokuni S, Nakamura H, Yodoi J, Kumagai S. Thioredoxin as a biomarker for oxidative stress in patients with rheumatoid arthritis. *Mol Immunol* 2002 February;38(10):765-72.

(66) Leaver SK, MacCallum NS, Pingle V, Hacking MB, Quinlan GJ, Evans TW, Burke-Gaffney A. Increased plasma thioredoxin levels in patients with sepsis: positive association with macrophage migration inhibitory factor. *Intensive Care Med* 2010 February;36(2):336-41.

(67) Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. *J Biol Chem* 2008 January 18;283(3):1622-7.

(68) Lehel C, Olah Z, Petrovics G, Jakab G, Anderson WB. Influence of various domains of protein kinase C epsilon on its PMA-induced translocation from the Golgi to the plasma membrane. *Biochem Biophys Res Commun* 1996 June 5;223(1):98-103.

(69) Ambruso DR, Ellison M, Thurman GW, Leto TL. Peroxiredoxin 6 translocates to the plasma membrane during neutrophil activation and is required for optimal NADPH oxidase activity. *Biochim Biophys Acta* 2011 December 8.

(70) Chang JW, Lee SH, Lu Y, Yoo YJ. Transforming growth factor-beta1 induces the non-classical secretion of peroxiredoxin-I in A549 cells. *Biochem Biophys Res Commun* 2006 June 23;345(1):118-23.

(71) Chang JW, Lee SH, Jeong JY, Chae HZ, Kim YC, Park ZY, Yoo YJ. Peroxiredoxin-I is an autoimmunogenic tumor antigen in non-small cell lung cancer. *FEBS Lett* 2005 May 23;579(13):2873-7.

(72) Yoshida S, Katoh T, Tetsuka T, Uno K, Matsui N, Okamoto T. Involvement of thioredoxin in rheumatoid arthritis: its costimulatory roles in the TNF-alpha-induced production of IL-6 and IL-8 from cultured synovial fibroblasts. *J Immunol* 1999 July 1;163(1):351-8.

(73) Hunter-Lavin C, Davies EL, Bacelar MM, Marshall MJ, Andrew SM, Williams JH. Hsp70 release from peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 2004 November 12;324(2):511-7.

(74) Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002 April 26;277(17):15028-34.

(75) Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, Devera ME, Liang X, Tor M, Billiar T. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol Rev* 2007 December;220:60-81.

(76) Westermann P, Knoblich M, Maier O, Lindschau C, Haller H. Protein kinase C bound to the Golgi apparatus supports the formation of constitutive transport vesicles. *Biochem J* 1996 December 1;320 (Pt 2):651-8.

(77) Staubach S, Razawi H, Hanisch FG. Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7. *Proteomics* 2009 May;9(10):2820-35.

(78) Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, Neve EP, Scheynius A, Gabrielsson S. Exosomes with immune modulatory features are present in human breast milk. *J Immunol* 2007 August 1;179(3):1969-78.

(79) Gonzalez-Begne M, Lu B, Han X, Hagen FK, Hand AR, Melvin JE, Yates JR. Proteomic analysis of human parotid gland exosomes by multidimensional protein identification technology (MudPIT). *J Proteome Res* 2009 March;8(3):1304-14.

(80) Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* 2003 February 6;91(3A):7A-11A.

(81) Lu Y, Wahl LM. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. *J Immunol* 2005 October 15;175(8):5423-9.

(82) Blumberg J. Use of biomarkers of oxidative stress in research studies. *J Nutr* 2004 November;134(11):3188S-9S.

(83) Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. *J Am Coll Cardiol* 2006 April 18;47(8 Suppl):C13-C18.

(84) Jo H, Song H, Mowbray A. Role of NADPH oxidases in disturbed flow- and. *Antioxid Redox Signal* 2006 September;8(9-10):1609-19.

(85) Qu A, Jiang C, Xu M, Zhang Y, Zhu Y, Xu Q, Zhang C, Wang X. PGC-1alpha attenuates neointimal formation via inhibition of vascular smooth muscle cell migration in the injured rat carotid artery. *Am J Physiol Cell Physiol* 2009 September;297(3):C645-C653.

(86) Popowich DA, Vavra AK, Walsh CP, Bhikhapurwala HA, Rossi NB, Jiang Q, Aalami OO, Kibbe MR. Regulation of reactive oxygen species by p53: implications for nitric oxide-mediated apoptosis. *Am J Physiol Heart Circ Physiol* 2010 June;298(6):H2192-H2200.

(87) Sahaf B, Rosen A. Secretion of 10-kDa and 12-kDa thioredoxin species from blood monocytes and transformed leukocytes. *Antioxid Redox Signal* 2000;2(4):717-26.

(88) Hess A, Wijayanti N, Neuschafer-Rube AP, Katz N, Kietzmann T, Immenschuh S. Phorbol ester-dependent activation of peroxiredoxin I gene expression via a protein

kinase C, Ras, p38 mitogen-activated protein kinase signaling pathway. *J Biol Chem* 2003 November 14;278(46):45419-34.

(89) Cha MK, Kim IH. Thioredoxin-linked peroxidase from human red blood cell: evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cell. *Biochem Biophys Res Commun* 1995 December 26;217(3):900-7.

(90) Martinez-Pinna R, Ramos-Mozo P, Madrigal-Matute J, Blanco-Colio LM, Lopez JA, Calvo E, Camafeita E, Lindholt JS, Meilhac O, Delbosc S, Michel JB, de Ceniga MV, Egido J, Martin-Ventura JL. Identification of peroxiredoxin-1 as a novel biomarker of abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol* 2011 April;31(4):935-43.

(91) Martinez-Pinna R, Lindholt JS, Blanco-Colio LM, Dejouvencel T, Madrigal-Matute J, Ramos-Mozo P, Vega de CM, Michel JB, Egido J, Meilhac O, Martin-Ventura JL. Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAAs). A potential link of oxidative stress with AAA evolution. *Atherosclerosis* 2010 June 1.

(92) Okuda M, Inoue N, Azumi H, Seno T, Sumi Y, Hirata K, Kawashima S, Hayashi Y, Itoh H, Yodoi J, Yokoyama M. Expression of glutaredoxin in human coronary arteries: its potential role in antioxidant protection against atherosclerosis. *Arterioscler Thromb Vasc Biol* 2001 September;21(9):1483-7.

(93) Takagi Y, Gon Y, Todaka T, Nozaki K, Nishiyama A, Sono H, Hashimoto N, Kikuchi H, Yodoi J. Expression of thioredoxin is enhanced in atherosclerotic plaques and during neointima formation in rat arteries. *Lab Invest* 1998 August;78(8):957-66.

(94) Nishihira K, Yamashita A, Imamura T, Hatakeyama K, Sato Y, Nakamura H, Yodoi J, Ogawa H, Kitamura K, Asada Y. Thioredoxin in coronary culprit lesions: possible relationship to oxidative stress and intraplaque hemorrhage. *Atherosclerosis* 2008 December;201(2):360-7.

(95) Furman C, Rundlof AK, Larigauderie G, Jaye M, Bricca G, Copin C, Kandoussi AM, Fruchart JC, Arner ES, Rouis M. Thioredoxin reductase 1 is upregulated in atherosclerotic plaques: specific induction of the promoter in human macrophages by oxidized low-density lipoproteins. *Free Radic Biol Med* 2004 July 1;37(1):71-85.

(96) Turoczi T, Chang VW, Engelman RM, Maulik N, Ho YS, Das DK. Thioredoxin redox signaling in the ischemic heart: an insight with transgenic mice overexpressing Trx1. *J Mol Cell Cardiol* 2003 June;35(6):695-704.

(97) Samuel SM, Thirunavukkarasu M, Penumathsa SV, Koneru S, Zhan L, Maulik G, Sudhakaran PR, Maulik N. Thioredoxin-1 gene therapy enhances angiogenic signaling and reduces ventricular remodeling in infarcted myocardium of diabetic rats. *Circulation* 2010 March 16;121(10):1244-55.

(98) Adluri RS, Thirunavukkarasu M, Zhan L, Akita Y, Samuel SM, Otani H, Ho YS, Maulik G, Maulik N. Thioredoxin 1 enhances neovascularization and reduces ventricular remodeling during chronic myocardial infarction: a study using thioredoxin 1 transgenic mice. *J Mol Cell Cardiol* 2011 January;50(1):239-47.

(99) Yamamoto M, Yang G, Hong C, Liu J, Holle E, Yu X, Wagner T, Vatner SF, Sadoshima J. Inhibition of endogenous thioredoxin in the heart increases oxidative stress and cardiac hypertrophy. *J Clin Invest* 2003 November;112(9):1395-406.

(100) Yang Y, Ago T, Zhai P, Abdellatif M, Sadoshima J. Thioredoxin 1 negatively regulates angiotensin II-induced cardiac hypertrophy through upregulation of miR-98/let-7. *Circ Res* 2011 February 4;108(3):305-13.

(101) Yoshioka J, Schulze PC, Cupesi M, Sylvan JD, MacGillivray C, Gannon J, Huang H, Lee RT. Thioredoxin-interacting protein controls cardiac hypertrophy through regulation of thioredoxin activity. *Circulation* 2004 June 1;109(21):2581-6.

(102) Lowenstein CJ. Exogenous thioredoxin reduces inflammation in autoimmune myocarditis. *Circulation* 2004 September 7;110(10):1178-9.

(103) Billiet L, Furman C, Larigauderie G, Copin C, Brand K, Fruchart JC, Rouis M. Extracellular human thioredoxin-1 inhibits lipopolysaccharide-induced interleukin-1beta expression in human monocyte-derived macrophages. *J Biol Chem* 2005 December 2;280(48):40310-8.

(104) Liu W, Nakamura H, Shioji K, Tanito M, Oka S, Ahsan MK, Son A, Ishii Y, Kishimoto C, Yodoi J. Thioredoxin-1 ameliorates myosin-induced autoimmune myocarditis by suppressing chemokine expressions and leukocyte chemotaxis in mice. *Circulation* 2004 September 7;110(10):1276-83.

(105) Aota M, Matsuda K, Isowa N, Wada H, Yodoi J, Ban T. Protection against reperfusion-induced arrhythmias by human thioredoxin. *J Cardiovasc Pharmacol* 1996 May;27(5):727-32.

(106) Hattori I, Takagi Y, Nakamura H, Nozaki K, Bai J, Kondo N, Sugino T, Nishimura M, Hashimoto N, Yodoi J. Intravenous administration of thioredoxin decreases brain damage following transient focal cerebral ischemia in mice. *Antioxid Redox Signal* 2004 February;6(1):81-7.

(107) Koneru S, Penumathsa SV, Thirunavukkarasu M, Zhan L, Maulik N. Thioredoxin-1 gene delivery induces heme oxygenase-1 mediated myocardial preservation after chronic infarction in hypertensive rats. *Am J Hypertens* 2009 February;22(2):183-90.

(108) Miyamoto S, Sakamoto T, Soejima H, Shimomura H, Kajiwara I, Kojima S, Hokamaki J, Sugiyama S, Yoshimura M, Ozaki Y, Nakamura H, Yodoi J, Ogawa H. Plasma thioredoxin levels and platelet aggregability in patients with acute myocardial infarction. *Am Heart J* 2003 September;146(3):465-71.

(109) Hokamaki J, Kawano H, Soejima H, Miyamoto S, Kajiwara I, Kojima S, Sakamoto T, Sugiyama S, Yoshimura M, Nakamura H, Yodoi J, Ogawa H. Plasma thioredoxin levels in patients with unstable angina. *Int J Cardiol* 2005 March 18;99(2):225-31.

(110) Wu Y, Yang L, Zhong L. Decreased serum levels of thioredoxin in patients with coronary artery disease plus hyperhomocysteinemia is strongly associated with the disease severity. *Atherosclerosis* 2010 September;212(1):351-5.

(111) Wahlgren CM, Pekkari K. Elevated thioredoxin after angioplasty in peripheral arterial disease. *Eur J Vasc Endovasc Surg* 2005 March;29(3):281-6.

(112) Wijayanti N, Naidu S, Kietzmann T, Immenschuh S. Inhibition of phorbol ester-dependent peroxiredoxin I gene activation by lipopolysaccharide via phosphorylation of RelA/p65 at serine 276 in monocytes. *Free Radic Biol Med* 2008 February 15;44(4):699-710.

(113) Choi MH, Lee IK, Kim GW, Kim BU, Han YH, Yu DY, Park HS, Kim KY, Lee JS, Choi C, Bae YS, Lee BI, Rhee SG, Kang SW. Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* 2005 May 19;435(7040):347-53.

(114) Rhee SG, Kang SW, Jeong W, Chang TS, Yang KS, Woo HA. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Curr Opin Cell Biol* 2005 April;17(2):183-9.

(115) Ihida-Stansbury K, McKean DM, Gebb SA, Martin JF, Stevens T, Nemenoff R, Vaughn J, Lane K, Loyd J, Wheeler L, Morrell NW, Ivy D, Jones PL. Regulation and functions of the paired-related homeobox gene PRX1 in pulmonary vascular development and disease. *Chest* 2005 December;128(6 Suppl):591S.

(116) Kisucka J, Chauhan AK, Patten IS, Yesilaltay A, Neumann C, Van Etten RA, Krieger M, Wagner DD. Peroxiredoxin1 prevents excessive endothelial activation and early atherosclerosis. *Circ Res* 2008 September 12;103(6):598-605.

(117) Park JG, Yoo JY, Jeong SJ, Choi JH, Lee MR, Lee MN, Lee JH, Kim HC, Jo H, Yu DY, Kang SW, Rhee SG, Lee MH, Oh GT. Peroxiredoxin 2 Deficiency Exacerbates Atherosclerosis in Apolipoprotein E-Deficient Mice. *Circ Res* 2011 August 11.

(118) Kang SW, Rhee SG, Chang TS, Jeong W, Choi MH. 2-Cys peroxiredoxin function in intracellular signal transduction: therapeutic implications. *Trends Mol Med* 2005 December;11(12):571-8.

(119) Li W, Febbraio M, Reddy SP, Yu DY, Yamamoto M, Silverstein RL. CD36 participates in a signaling pathway that regulates ROS formation in murine VSMCs. *J Clin Invest* 2010 November;120(11):3996-4006.

(120) Matsushima S, Ide T, Yamato M, Matsusaka H, Hattori F, Ikeuchi M, Kubota T, Sunagawa K, Hasegawa Y, Kurihara T, Oikawa S, Kinugawa S, Tsutsui H. Overexpression of mitochondrial peroxiredoxin-3 prevents left ventricular remodeling and failure after myocardial infarction in mice. *Circulation* 2006 April 11;113(14):1779-86.

FIGURE LEGENDS

Figure 1. Schematic diagram showing the maintenance of the cellular redox homeostasis by the NADPH oxidase, GSH and the TRX system. PRDXox-oxydized PRDX; PRDXred-reduced PRDX; TRX-S_2-oxydized TRX; TRX-SH_2- reduced TRX; GR-GSH reductase; GPx-GSH peroxidase.

Figure 2. Schematic diagram showing PRDX1/TRX main cellular location under non-stress conditions (left), under oxidative stress (right) and trafficking mechanisms (right).

FIGURE 1

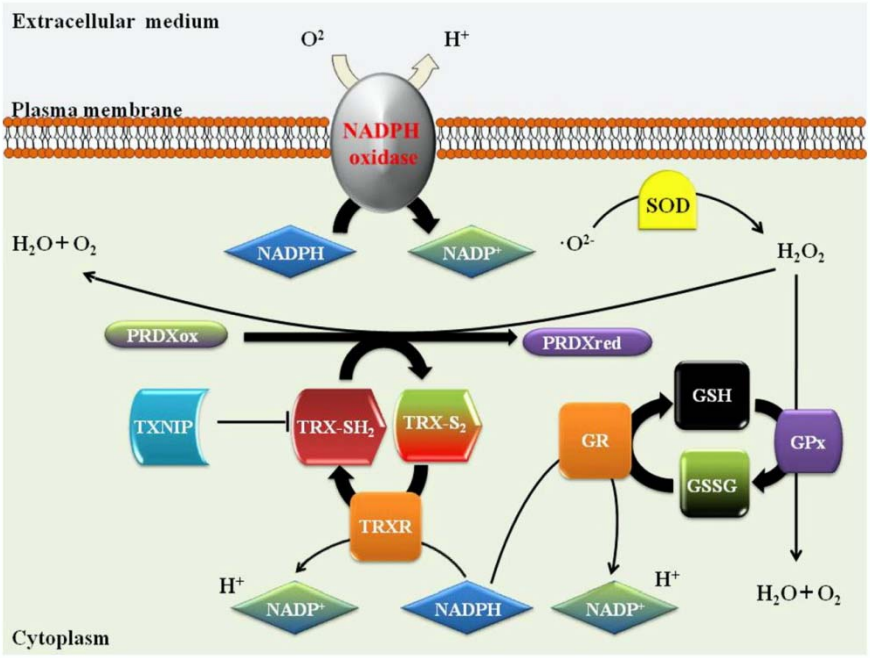
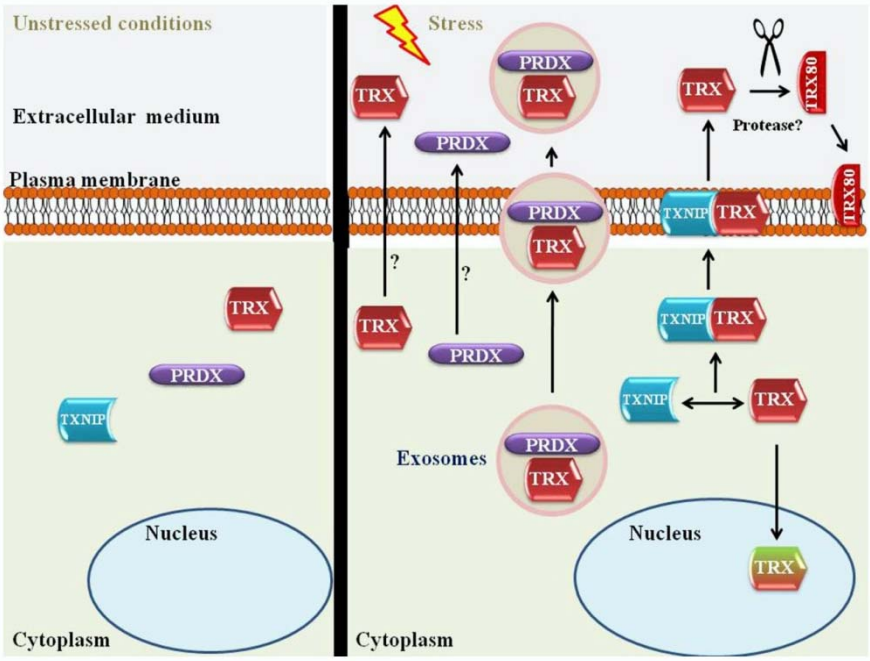


FIGURE 2



IX. ANEXO C

Atherosclerosis 220 (2012) 552–556

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Increased plasma levels of NGAL, a marker of neutrophil activation, in patients with abdominal aortic aneurysm

Priscila Ramos-Mozo[a], Julio Madrigal-Matute[a], Melina Vega de Ceniga[b], Luis Miguel Blanco-Colio[a], Olivier Meilhac[c], Laurent Feldman[c], Jean-Baptiste Michel[c], Paula Clancy[d], Jonathan Golledge[d], Paul E. Norman[e], Jesus Egido[a], José Luis Martin-Ventura[a,*]

[a] Vascular Research Lab, IIS, Fundación Jimenez Diaz, Autonoma University, Madrid, Spain
[b] Vascular Surgery, Hospital Galdakao-Usansolo, Bilbao, Spain
[c] Inserm, U698, Univ Paris 7, CHU X-Bichat, Paris, France
[d] Vascular Biology Unit, School of Medicine and Dentistry, James Cook University, Townsville 4811, Australia
[e] School of Surgery, University of Western Australia, Perth, Australia

ARTICLE INFO

Article history:
Received 18 May 2011
Received in revised form 26 October 2011
Accepted 16 November 2011
Available online 25 November 2011

Keywords:
Abdominal aortic aneurysm
Biomarkers
Polymorphonuclear neutrophils
Neutrophil gelatinase-associated lipocalin
Intraluminal thrombus

ABSTRACT

Objective: Neutrophil gelatinase-associated lipocalin (NGAL) plasma concentrations have been associated with cardiovascular diseases. We aimed to assess the association of NGAL with abdominal aortic aneurysm (AAA).

Methods: NGAL concentrations were analyzed by Western blotting in conditioned medium of polymorphonuclear neutrophils (PMNs) from AAA patients ($n = 22$) and controls ($n = 11$), and also in aortic biopsies from AAA patients and healthy controls ($n = 10$). Plasma NGAL concentrations were measured by ELISA in three groups of subjects from France ($n = 60$), Spain ($n = 75$) and Australia ($n = 100$) and associated with AAA presence and growth.

Results: PMNs isolated from AAA patients secreted significantly greater amounts of NGAL than PMNs from controls. Luminal thrombus released large amounts of NGAL compared to abluminal AAA thrombus, AAA wall and healthy aortic media. Plasma NGAL concentrations were significantly higher in patients with AAA than controls from France [115 (78–200) vs. 94 (72–114) ng/ml, $p < 0.001$]. NGAL plasma concentrations in AAA patients from Spain correlated with other markers of thrombus activity (plasmin–antiplasmin complexes and D-dimer). Furthermore, a positive correlation between plasma NGAL and retrospective AAA growth (rho = 0.4, $p = 0.01$) was observed, which remained significant after adjusting for other risk factors. Plasma NGAL was only weakly associated with prospective growth in both Spanish and Australian patients.

Conclusions: NGAL is released by PMNs and by the luminal part of AAA thrombus. NGAL plasma levels were increased in AAA patients compared with healthy subjects and correlated with retrospective AAA growth. Further studies in larger subjects groups are needed to confirm the association between NGAL and AAA presence and growth.

1. Introduction

Abdominal aortic aneurysm (AAA) is an important health problem in the elderly. In cross-sectional studies the prevalence of AAA varies from 3% to 8% in men aged >65 years. In elderly men AAA may cause as much as 2–3% of all deaths [1].

Human AAAs are characterized by the presence of intraluminal thrombus (ILT), an important source of proteolytic and oxidative enzymes implicated in AAA [2,3]. Polymorphonuclear neutrophils (PMNs), along with platelets and red blood cells, are particularly abundant within the luminal layer of AAA thrombus. PMNs may contribute to two main mechanisms of AAA evolution, namely medial destruction and adventitial immune-inflammatory processes [4,5]. A role for PMNs in the pathogenesis of AAA is supported by recent studies in animal models of AAA [6,7]. PMN depletion is able to inhibit experimental AAA formation [7] and short-term preoperative doxycycline therapy improves the proteolytic balance in human AAA, possibly through limiting aortic wall neutrophil

* Corresponding author at: Vascular Research Lab, IIS, Fundación Jiménez Diaz, Autonoma University, Av. Reyes Católicos 2, 28040 Madrid, Spain.
Tel.: +34 915504800; fax: +34 915442636.
E-mail address: jlmartin@fjd.es (J.L. Martin-Ventura).

0021-9150/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2011.11.023

infiltration [8]. This data highlights the potential value of analyzing PMNs as a source of AAA biomarkers.

Neutrophil gelatinase-associated lipocalin (NGAL) was originally isolated as a neutrophil-derived protein [9] and these cells have been found to represent an important source for circulating NGAL during inflammation [10]. Plasma NGAL concentrations have been correlated to cardiovascular risk factors in patients with asymptomatic atherosclerosis [11]. Serum NGAL concentrations have been reported to be significantly higher in patients with coronary artery disease (CAD) and chronic heart failure (CHF), and correlated with the severity of the disease [12,13]. Very recently, NGAL plasma concentrations have been correlated with markers of renal function (e.g. creatinine) in patients with carotid atherosclerosis, suggesting that NGAL levels could reflect a potential link between both pathologies [14]. Although the presence of NGAL has been previously observed in human AAA tissue [15], no studies have analyzed the association of circulating NGAL concentrations with AAA. Our hypothesis was that circulating NGAL concentrations would reflect leukocyte activation in patients with AAA. To assess this hypothesis, we first analyzed NGAL release by PMNs and human AAA tissue. Secondly, we measured NGAL concentrations in the plasma of three groups of patients and related them to the presence, size and growth of AAAs.

2. Materials and methods

2.1. AAA patients

2.1.1. French subjects

Plasma was obtained from patients ($n = 30$) undergoing AAA surgery and enrolled in the RESAA protocol (REflet Sanguin de l'évolutivité des Anévrysmes de l'Aorte abdominale) (Table 1 online). All patients gave informed written consent, and the protocol was approved by a French ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomedicale, CCPRB Paris-Cochin, approval no 2095) [16].

Control subjects were a sub-group of the BIOmarkers of CORonary Events (BIOCORE) – 1 study (ClincalTrials.gov identifier: NCT00430820). Subjects ($n = 30$) selected required a normal coronary angiography and had no documented AAA. Subjects were age and sex matched with the AAA patients.

2.1.2. Spanish subjects

Plasma samples were obtained from 75 patients with infrarenal AAAs (AAA size > 30 mm) recruited from Galdakao Hospital (Table 2 online). More information regarding these patients can be found in the online supplement data. We registered the maximum aortic diameter at the time of blood sample harvest and AAA growth in the previous 12 months (retrospective 1-year AAA growth = AAA diameter at the time of blood sample harvest – AAA diameter 1 year previously). We prospectively followed the patients with annual ultrasound scanning (if the baseline AAA diameter was 3–3.9 cm), or with six-monthly abdominal contrast CT scan (if the baseline AAA diameter was 4–4.9 cm). We calculated the prospective 1-year AAA growth as: AAA diameter 1 year after blood sample harvest – AAA diameter at the time of blood sample harvest.

2.1.3. Australian subjects

Australian subjects were recruited from the Health in Men Study (HIMS), which has been described in detail in a previous publication [17]. This cohort includes men who took part in a trial of ultrasound screening for AAA of whom a sub-set later returned for blood sampling. We included 100 men with AAA (aortic diameter ≥ 30 mm) for whom ultrasound surveillance of their aneurysm was available (Table 3 online). The annual growth rates of AAAs were assessed by taking into account all diameters measured during follow-up and calculating time-weighted average growth rates for each patient [18].

All studies were approved by the corresponding Scientific Ethical Committees, and informed consent from the patients was obtained.

2.2. Neutrophil-conditioned media

Blood samples were obtained from an additional 22 AAA patients and 11 controls matched by age and sex from Spain. Neutrophils were obtained from venous blood by Ficoll-Paque (Amersham, Piscataway, NJ, USA) followed by 6% dextran sedimentation and hypo-osmotic lysis of residual erythrocytes. PMNs isolation was assessed by flow cytometry to make sure that they were not contaminated by others cell types. The analysis of CD16 from PMNs shows a strong fluorescence signal in all samples (not shown). One million PMNs isolated from blood samples were cultured in RPMI medium and proteins released were obtained after 30 min of culture.

2.3. AAA tissue and tissue-conditioned media

Ten AAA thrombus samples were collected during surgical repair and dissected into luminal and abluminal parts (respectively at the interface with circulating blood and with the remaining media). AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol [16]. Ten control aortas were sampled from dead organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal and devoid of early atheromatous lesions. Different layers of AAA thrombus and wall, as well as healthy walls, were cut into small pieces (5 mm^2) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 h at 37 °C (6 ml/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation at 3000 × g for 10 min at 20 °C.

2.4. Western blotting

Equal amounts (5 μL) of conditioned medium (previously normalized to tissue weight: 1 g/6 mL) were electrophoresed as described [19]. The membranes were incubated with monoclonal antibodies against lipocalin-2/NGAL antibody (1:1000; R&D system). After they were incubated with HRP (horseradish peroxidase)-conjugated anti-goat IgG antibody (Santa Cruz) at a dilution of 1:2500. The proteins were then detected by enhanced chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences) and evaluated by densitometry (Quantity One; Bio-Rad Laboratories). Pre-stained protein markers (PageRuler™ Prestained Protein Ladder; Fermentas) were used for molecular mass determinations.

2.5. ELISA

We quantified the soluble concentrations of NGAL (Bioporto diagnostics) in plasma and MPO (HYCULT) in conditioned media with commercial kits, following manufacturer's instructions.

2.6. Determination of cf-DNA

Cell-free DNA (cf-DNA) was considered as reflecting neutrophil extracellular trap (NET) formation. Cf-DNA concentration was determined in the medium conditioned by neutrophils incubated for 30 min at 37 °C without stimulation using Quant-it™ Picogreen® ds DNA Reagent (Invitrogen, France). Supernatants

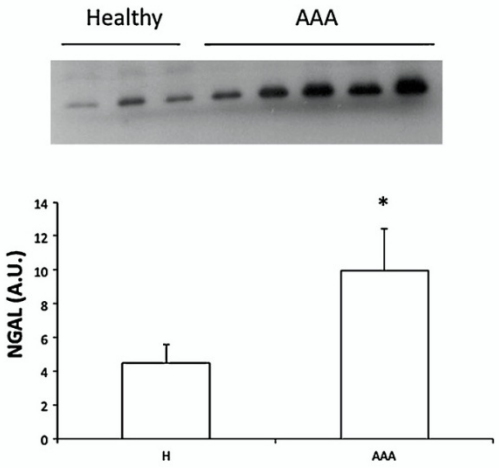


Fig. 1. NGAL levels in PMN-conditioned media of AAA patients and controls. Western-blot and quantification of NGAL levels in AAA patients ($n = 22$) and controls (H, $n = 11$). *$p < 0.05$ vs. controls. AU = arbitrary units.

obtained after centrifugation (1500 × *g*, 5 min. 20 °C) were diluted 1:10 in TE buffer (200 mM Tris–HCl, 20 mM EDTA, pH 7.5, 100 μL final) before addition of 100 μL Picogreen® dsDNA reagent. The fluorescence was then measured using a microplate reader (excitation 480 nm, emission 520 nm) after mixing and incubation for 5 min at room temperature in the dark.

2.7. Statistical analysis

In vitro results are expressed as mean ± standard error. The Wilcoxon paired test was used to analyze differences in NGAL levels between luminal, abluminal and media supernatants of the same subjects. NGAL levels in AAA patients and controls are expressed as median (interquartile ranges) and were analyzed by the Mann–Whitney *U*-test. Univariate association was performed by Spearman correlation test. Multivariate linear regression analysis was conducted with AAA size or growth as dependent variables, including in the model the traditional risk factors and those variables that were significant in the univariate analysis. The statistical analysis was performed using the SPSS 15.0 software and $p < 0.05$ as significant.

3. Experimental results

3.1. NGAL is released by PMN from AAA patients

The release of NGAL from PMNs isolated from AAA patients was significantly greater than from PMNs isolated from controls (9.9 ± 2.6 vs. 4.5 ± 1.2 a.u., $p < 0.05$, Fig. 1). Similarly, MPO and Cf-DNA levels were also increased in conditioned media of PMN of AAA patients compared to controls assessed (96.8 ± 16.8 vs. 12.5 ± 5 ng/ml and 241 ± 13 vs. 225 ± 9 ng/ml, respectively, $p < 0.05$ for both, not shown). A strong correlation was observed between NGAL and MPO or Cf-DNA ($r = 0.5$ for both, $p < 0.05$). These results suggest a potential activated state of circulating PMN in AAA patients.

3.2. NGAL is released by the luminal part of AAA thrombus

Since the luminal layer of ILT of human AAA is enriched in PMN, we analyzed the amount of NGAL in the conditioned media of explants of ILT, AAA wall, and healthy aortic wall. The amount of NGAL released was greatest from explants of the luminal layer of the ILT as compared to both abluminal thrombus and pathological AAA media (21.1 ± 7.9 vs. 11.1 ± 6.2 vs. 8.3 ± 4.9 a.u., $p < 0.05$ for both, Fig. 2). Moreover, pathological media also release more NGAL than healthy media, where NGAL was almost absent (8.3 ± 4.9 vs. 2.1 ± 2.1 a.u., $p < 0.05$, Fig. 2).

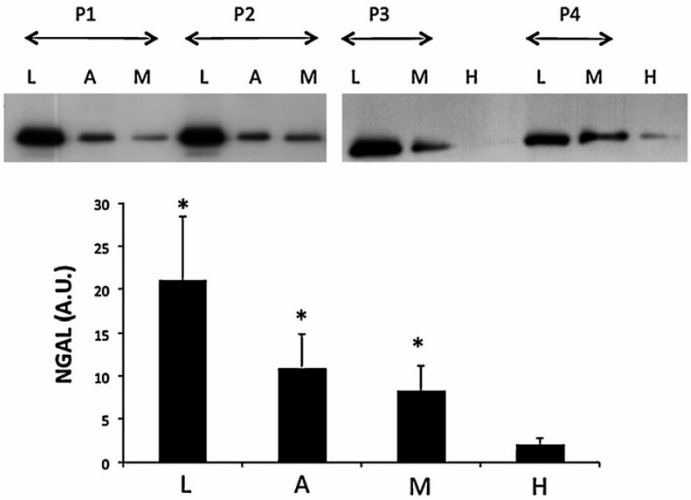


Fig. 2. NGAL levels in AAA and healthy tissue-conditioned media. Western-blot and quantification of NGAL levels in luminal (L), abluminal (A) and media (M) of AAA ($n = 10$), as well as in healthy media (H, $n = 10$). *$p < 0.05$ L vs. A and M, A vs. M and M vs. H. Representative western blot of 4 thrombus of patients (P1–P4) and 2 healthy aortas. AU = arbitrary units.

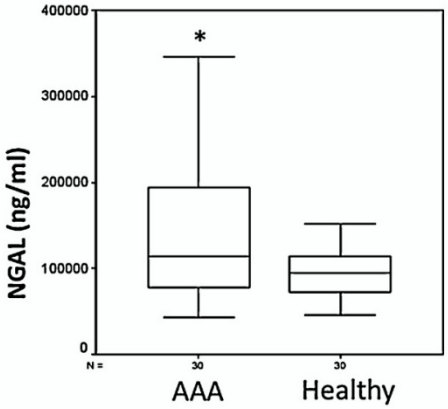


Fig. 3. NGAL plasma levels in AAA patients and healthy subjects. ELISA of NGAL in patients with AAA ($n = 30$) and healthy controls ($n = 30$) from France, *$p < 0.001$ vs. controls.

3.3. Increased NGAL plasma levels in AAA patients and correlation with clinical and biological determinants of AAA activity

In French subjects circulating NGAL levels were significantly higher in AAA patients (AAA size > 50 mm, $n = 30$) compared to control subjects ($n = 30$) [115 (78–200) vs. 94 (72–114) ng/ml, $p < 0.001$, Fig. 3]. This data was confirmed in 30 AAA patients (AAA size = 30–50 mm) and 30 age- and sex-matched control subjects from Spain [90 (79–113) vs. 80 (63–106), $p < 0.05$, not shown]. To try to address the potential role of NGAL in AAA pathogenesis, we analyzed its association with AAA size in another group of plasma samples of AAA patients (AAA size > 30 mm, $n = 75$) from Spain [109 (75–124) ng/ml]. Interestingly, NGAL levels and AAA diameter were significantly correlated ($r = 0.27$, $p < 0.05$), although significance disappears after multivariate analysis. In addition we analyzed the potential correlation of NGAL with markers associated to thrombus activity, such as myeloperoxidase (MPO), plasmin–antiplasmin complexes (PAP) and D-dimers (DD). NGAL levels correlated with MPO, PAP and DD (0.23, 0.25 and 0.28, respectively, $p < 0.05$ for all). Furthermore, NGAL levels also correlated with leukocyte count and creatinine ($r = 0.24$ and 0.56 respectively, $p < 0.05$ for both). In a subset of patients ($n = 40$) with data on retrospective and prospective 1-year growth, NGAL also correlated with retrospective 1-year growth ($r = 0.4$, $p = 0.01$) and a non-significant trend was observed with prospective 1-year growth ($r = 0.2$, $p = 0.14$). The correlation between NGAL levels and retrospective growth rate in the Spanish population persisted after adjustment for sex, age, dyslipidemia, current smoking, arterial hypertension, heart disease, creatinine and statin use (Table 1).

In order to further assess the potential prognostic value of NGAL we evaluated its plasma concentration in another group of 100 patients with AAAs from Australia (median initial AAA diameter 37.5, interquartile range 35.3–41.3 mm) followed for a median of 5.5 (interquartile range 2.5–6.0) years by a median of 7 (interquartile range 4–9) ultrasound scans. NGAL plasma concentration was weakly associated with averaged yearly AAA growth ($r = 0.129$, $p = 0.201$). Median weighted yearly AAA growths for patients with NGAL above and less than or equal to median concentration (≥53.30 ng/ml) were 1.7 (1.0–3.0) and 1.4 (0.8–2.1) mm/year, respectively, $p = 0.202$. A close correlation between plasma NGAL and creatinine was observed in these patients ($r = 0.409$, $p < 0.0001$).

Table 1
Multivariate analysis in the Spanish group.

Model	Coefficients[a]				
	Unstandardized coefficients		Standardized coefficients	t	Sig.
	B	Std. error	Beta		
(Constant)	−1.094	5.982		−0.183	0.856
N-GAL	2.129E−5	0.000	0.456	2.421	0.022
Sex	−1.654	2.184	−0.124	−0.758	0.455
Age	0.058	0.070	0.152	0.836	0.410
Dyslipemia	−0.635	1.634	−0.105	−0.389	0.700
Smoking	1.407	0.963	0.232	1.460	0.155
HTA	1.481	1.024	0.237	1.446	0.159
Heart disease	−0.252	1.214	−0.039	−0.207	0.837
Creatinine	−2.385	2.207	−0.208	−1.081	0.289
Statin use	0.154	1.592	0.026	0.007	0.924

[a] Dependent variable: retrospective growth.

4. Discussion

In the present study, we have shown that NGAL is released by circulating PMNs and by ILT of AAA patients. Furthermore, NGAL plasma concentrations were higher in subjects with AAA compared to age-and sex-matched healthy controls. Furthermore, we have observed that NGAL levels positively correlated with AAA retrospective growth.

PMNs activation can contribute to the main mechanisms of AAA evolution, namely ILT formation, oxidative stress, proteolytic degradation of the aortic media and adventitial inflammation. In this respect, previous studies support the importance of leukocyte activation in vascular diseases [11,20,21]. In CAD patients, systemic IL-8 and NGAL concentrations were significantly increased, indicating a primed state of circulating neutrophils in CAD [21]. In agreement, we have shown that NGAL and MPO and Cf-DNA levels are increased in PMN-conditioned media from AAA patients compared to healthy controls. Moreover, NGAL positively correlated with MPO and Cf-DNA in PMN-conditioned media, suggesting activation of circulating PMNs before entering the aneurysmal tissue. Interestingly, a previous report showed expression of NGAL in the luminal part of AAA [15]. In agreement, we have shown that NGAL levels were increased in conditioned media from explants of the luminal part of AAA-thrombus, probably reflecting the activation of PMNs entrapped within the ILT of human AAA. In this respect, we have previously shown increased levels of markers of neutrophil activation such as MPO and Cf-DNA both in AAA tissue-conditioned media and in plasma [22,23]. In the present paper, we have observed that circulating NGAL positively correlated with leukocyte count and MPO, further supporting the potential role of activated PMNs in the release of NGAL in AAA patients. Similarly, increased levels of PAP and DD were shown both in ILT conditioned media and plasma of AAA patients [24]. In the present paper, NGAL correlated with both, PAP and DD, suggesting that increased NGAL plasma levels observed in AAA patients could be a surrogate marker of the thrombus biological activity.

NGAL exists in monomeric and homo- and heterodimeric forms, the latter as a dimer with human neutrophil gelatinase, MMP-9 [10]. NGAL and gelatinase are in part physically separated, which may explain the existence of unassociated forms of both proteins in human neutrophils [25]. Although associated with neutrophil gelatinase, NGAL does not directly reflect the activity of gelatinase. In this respect, approximately 50% of the NGAL–gelatinase complex isolated from human neutrophils exists in a ternary complex with tissue inhibitor of metalloproteinase-1 (TIMP-1). Gelatinase in this ternary complex has a 10-fold lower activity when activated than the forms of gelatinase not associated with TIMP-1 [26]. There was a previous report showing expression of NGAL associated to

MMP9 in the luminal part of AAA tissue [15]. Moreover, we have previously shown that NGAL/MMP9 complexes were increased in the conditioned media of ILT of human AAA and in plasma [22]. NGAL/MMP9 is not associated with AAA size or growth in the Spanish cohort (unpublished observations). Surprisingly, we did not find any association between NGAL and MMP9/NGAL complexes in plasma suggesting that NGAL could have an independent role to that of MMP9. In this respect, the rodent analogues of NGAL are not associated with gelatinase, indicating that this well conserved lipocalin may have other important functions [10]. Among these, NGAL possesses bacteriostatic properties by binding bacterial siderophores, thereby preventing bacteria from retrieving iron from this source [27]. Thus, our results could suggest that recruitment of PMNs in the iron-rich ILT release NGAL as a protective response to increased bacteria present in AAA tissue [23]. In any case, our observational study can not exclude the possibility that increased NGAL levels could also be, at least in part, the consequence of a response to increased MMP9 levels.

Although NGAL was identified in human neutrophils [9], NGAL may also be released by epithelial cells, renal tubular cells and hepatocytes during inflammation or injury [10,26]. Moreover, NGAL is a promising biomarker of acute kidney injury and increased NGAL levels correlate with decreased renal function [28]. Although impaired renal function has been linked to poor outcome after AAA repair, little is known about the role of chronic renal failure (CRF) as a potential risk factor in patients with AAA. In this respect, we have previously described that CRF was associated with higher AAA growth rates and more rapid expansion (>4 mm/year) in a large cohort of 3–3.9 cm AAA patients [29]. In the Spanish cohort, creatinine was not associated with AAA size or growth. NGAL positively correlated with creatinine in the Spanish and in the Australian cohort. However, correlation between NGAL and AAA activity in the Spanish cohort remained significant after analyzing the creatinine levels, suggesting that NGAL could have a role as a biomarker of AAA activity independently to its potential role as a biomarker of renal disease. However, further studies in larger patient cohorts are needed to confirm this issue.

On the whole, we have shown that NGAL is released by circulating PMNs and ILT of AAA patients. Furthermore, NGAL plasma levels are increased in AAA patients and correlate with AAA growth, probably reflecting a potential activation of both resident and circulating leukocytes. The biological consequences of increased NGAL levels are potentially linked to their functions as modulators of immune response [27]. However, further studies are needed to clarify the role of NGAL in AAA.

Acknowledgements

The paper have been supported by grants from the Basque Government (2008111019), cvREMOD 091100, the European Community, FAD project (FP-7, HEALTH F2-2008-200647), the Spanish Ministerio de Ciencia y Tecnología (SAF 2010/21852), HF2007-0120, Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035) and EUS2008-03565. Research conducted by JG is supported by the NHMRC and Office of Health and Medical Research, Australia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.11.023.

References

[1] Sakalihasan N, Limet R, Defawe OD. Abdominal aortic aneurysm. Lancet 2005;365:1577–89.
[2] Swedenborg J, Eriksson P. The intraluminal thrombus as a source of proteolytic activity. Ann N Y Acad Sci 2006;1085:133–8.
[3] Michel JB, Martin-Ventura JL, Egido J, et al. Novel aspects of the pathogenesis of aneurysms of the abdominal aorta in humans. Cardiovasc Res 2011;90:18–27.
[4] Thompson RW, Geraghty PJ, Lee JK. Abdominal aortic aneurysms: basic mechanisms and clinical implications. Curr Probl Surg 2002;39:110–230.
[5] Michel JB, Thaunat O, Houard X, Meilhac O, Caligiuri G, Nicoletti A. Topological determinants and consequences of adventitial responses to arterial wall injury. Arterioscler Thromb Vasc Biol 2007;27:1259–68.
[6] Hannawa KK, Eliason JL, Woodrum DT, et al. L-selectin-mediated neutrophil recruitment in experimental rodent aneurysm formation. Circulation 2005;112:241–7.
[7] Eliason JL, Hannawa KK, Ailawadi G, et al. Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation. Circulation 2005;112:232–324.
[8] Lindeman JH, Abdul-Hussien H, van Bockel JH, Wolterbeek R, Kleemann R. Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells. Circulation 2009;119:2209–16.
[9] Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem 1993;268:10425–32.
[10] Kjeldsen L, Cowland JB, Borregaard N. Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. Biochim Biophys Acta 2000;1482:272–83.
[11] Elneihoum AM, Falke P, Hedblad B, Lindgarde F, Ohlsson K. Leukocyte activation in atherosclerosis: correlation with risk factors. Atherosclerosis 1997;131:79–84.
[12] Zografos T, Haliassos A, Korovesis S, Giazitzoglou E, Voridis E, Katritsis D. Association of neutrophil gelatinase-associated lipocalin with the severity of coronary artery disease. Am J Cardiol 2009;104:917–20.
[13] Yndestad A, Landrø L, Ueland T, et al. Increased systemic and myocardial expression of neutrophil gelatinase-associated lipocalin in clinical and experimental heart failure. Eur Heart J 2009;30:1229–36.
[14] Giaginis C, Zira A, Katsargyris A, Klonaris C, Theocharis S. Clinical implication of plasma neutrophil gelatinase-associated lipocalin (NGAL) concentrations in patients with advanced carotid atherosclerosis. Clin Chem Lab Med 2010;48:1035–41.
[15] Folkesson M, Kazi M, Zhu C, et al. Presence of NGAL/MMP-9 complexes in human abdominal aortic aneurysms. Thromb Haemost 2007;98:427–33.
[16] Caligiuri G, Rossignol P, Julia P, et al. Reduced immunoregulatory CD31+ T cells in patients with atherosclerotic abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 2006;26:618–23.
[17] Norman PE, Flicker L, Almeida OP, Hankey GJ, Hyde Z, Jamrozik K. Cohort profile the health in men study (HIMS). Int J Epidemiol 2009;38:48–52.
[18] Ferguson CD, Clancy P, Bourke B, et al. Association of statin prescription with small abdominal aortic aneurysm progression. Am Heart J 2010;159:307–13.
[19] Martinez-Pinna R, Lindholt JS, Blanco-Colio LM, et al. Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAAs). A potential link of oxidative stress with AAA evolution. Atherosclerosis 2010;212:333–8.
[20] Falke P, Elneihoum AM, Ohlsson K. Leukocyte activation: relation to cardiovascular mortality after cerebrovascular ischemia. Cerebrovasc Dis 2000;10:97–101.
[21] Paulsson J, Dadfar E, Held C, Jacobson SH, Lundahl J. Activation of peripheral and in vivo transmigrated neutrophils in patients with stable coronary artery disease. Atherosclerosis 2007;192:328–34.
[22] Houard X, Touat Z, Ollivier V, et al. Mediators of neutrophil recruitment in human abdominal aortic aneurysms. Cardiovasc Res 2009;82:532–41.
[23] Delbosc S, Alsac JM, Journe C, et al. Porphyromonas gingivalis participates in pathogenesis of human abdominal aortic aneurysm by neutrophil activation. proof of concept in rats. PLoS One 2011;6:e18679.
[24] Houard X, Rouzet F, Touat Z, et al. Topology of the fibrinolytic system within the mural thrombus of human abdominal aortic aneurysms. J Pathol 2007;212:20–8.
[25] Kjeldsen L, Sengeløv H, Lollike K, Nielsen MH, Borregaard N. Isolation and characterization of gelatinase granules from human neutrophils. Blood 1994;83:1640–9.
[26] Kolkenbrock H, Hecker-Kia A, Orgel D, Ulbrich N, Will H. Activation of progelatinase A and progelatinase A/TIMP-2 complex by membrane type 2-matrix metalloproteinase. Biol Chem 1997;378:71–6.
[27] Flo TH, Smith KD, Sato S, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature 2004;432:917–21.
[28] Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A, NGAL Meta-analysis Investigator Group. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis 2009;54:1012–24.
[29] Vega de Céniga M, Gómez R, Estallo L, Rodríguez L, Baquer M, Barba A. Growth rate and associated factors in small abdominal aortic aneurysms. Eur J Vasc Endovasc Surg 2006;31:231–6.

Arteriosclerosis, Thrombosis, and Vascular Biology
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®
Learn and Live℠

Proteomic Analysis of Polymorphonuclear Neutrophils Identifies Catalase as a Novel Biomarker of Abdominal Aortic Aneurysm: Potential Implication of Oxidative Stress in Abdominal Aortic Aneurysm Progression

Priscila Ramos-Mozo, Julio Madrigal-Matute, Roxana Martinez-Pinna, Luis Miguel Blanco-Colio, Juan Antonio Lopez, Emilio Camafeita, Olivier Meilhac, Jean-Baptiste Michel, Cesar Aparicio, Melina Vega de Ceniga, Jesus Egido and José Luis Martín-Ventura

Arterioscler Thromb Vasc Biol 2011, 31:3011-3019: originally published online September 22, 2011
doi: 10.1161/ATVBAHA.111.237537
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online ISSN: 1524-4636

Proteomic Analysis of Polymorphonuclear Neutrophils Identifies Catalase as a Novel Biomarker of Abdominal Aortic Aneurysm: Potential Implication of Oxidative Stress in Abdominal Aortic Aneurysm Progression

Priscila Ramos-Mozo, Julio Madrigal-Matute, Roxana Martinez-Pinna, Luis Miguel Blanco-Colio, Juan Antonio Lopez, Emilio Camafeita, Olivier Meilhac, Jean-Baptiste Michel, Cesar Aparicio, Melina Vega de Ceniga, Jesus Egido, José Luis Martín-Ventura

Objective—Polymorphonuclear neutrophils (PMNs) play a main role in abdominal aortic aneurysm (AAA) progression. We have analyzed circulating PMNs isolated from AAA patients and controls by a proteomic approach to identify proteins potentially involved in AAA pathogenesis.

Methods and Results—PMNs from 8 AAA patients (4 large AAA >5 cm and 4 small AAA 3–5 cm) and 4 controls were analyzed by 2D differential in-gel electrophoresis. Among differentially expressed spots, several proteins involved in redox balance were identified by mass spectrometry (eg, cyclophilin, thioredoxin reductase, catalase). Diminished catalase expression and activity were observed in PMNs from AAA patients compared with controls. In contrast, PMNs from AAA patients displayed higher H_2O_2 and myeloperoxidase levels than PMNs from controls. Moreover, a significant decrease in catalase mRNA levels was observed in PMNs after phorbol 12-myristate 13-acetate incubation. Catalase plasma levels were also decreased in large (n=47) and small (n=56) AAA patients compared with controls (n=34). We observed catalase expression in AAA thrombus and thrombus-conditioned medium, associated with PMN infiltration. Furthermore, increased H_2O_2 levels were observed in AAA thrombus-conditioned medium compared with the media layer.

Conclusion—Diminished catalase levels in circulating PMNs and plasma are observed in AAA patients, supporting an important role of oxidative stress in AAA evolution. (***Arterioscler Thromb Vasc Biol*. 2011;31:3011-3019.)**

Key Words: aneurysms ■ antioxidants ■ leukocytes

Abdominal aortic aneurysm (AAA) is an important health problem in elderly. In cross-sectional studies, the prevalence varies from 3% to 8%.[1] In elderly men, AAAs may cause as much as 2% to 3% of all deaths.[1] Because AAAs are usually asymptomatic, the present clinical challenges are early diagnosis and deciphering the biological mechanisms responsible for the progressive dilatation and final rupture to develop new diagnostic and therapeutic approaches.

Although polymorphonuclear neutrophils (PMNs) represent the major class of leukocytes, they have received little attention in atherothrombosis.[2,3] However, recent evidence is revealing a previously unappreciated role of PMN in experimental[4,5] and human[6,7] AAAs. PMNs can contribute to main mechanisms of AAA evolution, namely intraluminal thrombus (ILT) formation, oxidative stress, proteolytic degradation of the aortic media, and adventitial inflammation.[8] AAAs are characterized by the presence of a mural ILT-containing platelets, red blood cells (RBCs), and PMNs, particularly abundant within the luminal layer of human thrombus.[6–8] AAAs are also characterized by destructive connective tissue remodeling, including depletion of aortic elastin and fragmentation of medial elastic fibers.[9] Finally, inflammatory cells (macrophages and neutrophils) are also evident within the adventitia of human AAAs.[10] Interestingly, PMNs depletion is able to inhibit experimental AAA formation.[11] More recently, short-term preoperative doxycycline therapy improved the proteolytic balance in human AAA, presumably via an effect on aortic wall neutrophil content.[12]

These data highlight the potential interest of analyzing the PMNs proteome with the aim of identifying novel diagnostic and prognostic targets in AAA disease. Furthermore, identification of biomarkers could also afford novel pathogenic

Received on: January 24, 2011; final version accepted on: September 12, 2011.

From the Vascular Research Laboratory (P.R.-M., J.M.-M., R.M.-P., L.M.B.-C., J.E., J.L.M.-V.) and Vascular Surgery (C.A.), Instituto de Investigaciones Sanitarias, Fundación Jiménez Diaz, Autonoma University, Madrid, Spain; Unidad de Proteomica, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain (J.A.L., E.C.); Institut National de la Santé et de la Recherche Médicale, U698, Univ Paris 7, College Hospital Universitary, X-Bichat, Paris, France (O.M., J.-B.M.); Galdakao Hospital, Bilbao, Spain (M.V.d.C.).

Correspondence to José Luis Martín-Ventura, PhD, Vascular Research Laboratory, Fundación Jiménez Diaz-Instituto de Investigaciones Sanitarias, Autonoma University, Av Reyes Católicos 2, 28040 Madrid, Spain. E-mail jlmartin@fjd.es

Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.111.237537**

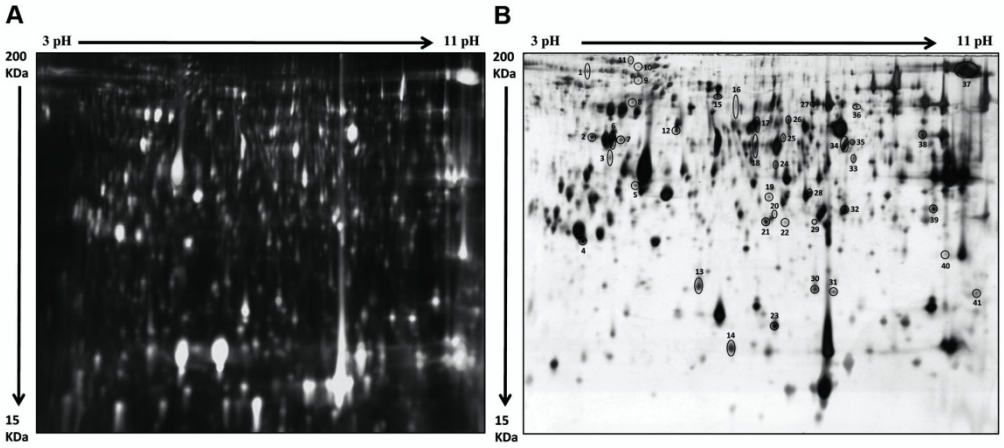


Figure 1. Differential in-gel electrophoresis analysis. Polymorphonuclear neutrophil (PMN) protein extracts of 8 abdominal aortic aneurysm (AAA) patients and 4 controls were labeled with the corresponding CyDye reagents, mixed, resolved on 6 independent differential in-gel electrophoresis gels, imaged (red, Cy3; green, Cy5; blue, Cy2) and analyzed with DeCyder software. **A,** A representative gel image from the differential in-gel electrophoresis experiment is shown. Proteins were resolved in the 3 to 11 (nonlinear) pH range on the first dimension and on 12% polyacrylamide gels on the second dimension. **B,** Spots showing statistically significant regulation between the 2 conditions were excised from silver-stained gels and identified by matrix-assisted laser desorption ionization mass spectrometry. Identified spots are numbered as in Supplemental Table II.

pathways and may thus open possibilities for pharmacological inhibition of growth, providing tools for monitoring this inhibition.[13] Until now, most studies have focused on the role of individual proteins related to different PMNs function/activities. Expression proteomic studies offer the possibility of finding out proteins that could be dysregulated in PMNs under pathological conditions. Previous studies analyzing expression proteome of PMNs have been recently thoroughly reviewed.[14] However, no studies have addressed the comparison of PMN proteome in vascular diseases. In the present study, we have performed a comparative 2D differential in-gel electrophoresis protein expression analysis of circulating PMNs isolated from AAA patients and controls.

Methods

AAA Patients

The blood samples used for proteomic analysis were obtained from 8 AAA patients before they underwent infrarenal AAA repair (n=4 large AAA, AAA diameter >5 cm) or patients who visited the vascular surgery department for follow-up assessment (n=4 small AAA, AAA diameter 3–5 cm). The control group consisted of 4 volunteers (AAA diameter <3 cm) without significant differences from the patient groups in age, sex, risk factors, or medications. Furthermore, 12 additional patients (6 large AAA and 6 small AAA) and 6 additional controls were used for further validation of the proteomic results. Finally, for further functional studies, PMNs were isolated from 10 additional controls and 10 patients (4 large AAA and 6 small AAA) and incubated with 50 μmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) or vehicle for 30 minutes (for hydrogen peroxide [H_2O_2]) or for 4 hours (for myeloperoxidase [MPO] and catalase/manganese superoxide dismutase [MnSOD] mRNA analysis) at 37°C.

In addition, 103 consecutive patients with an asymptomatic infrarenal AAA were recruited, and the AAA size at the time of plasma sample collection was registered (Supplemental Table I, available online at http://atvb.ahajournals.org). Similarly, 34 controls were recruited from a screening program, which is currently being performed among the population in the area under our care. They were randomly selected from the screened individuals with nondilated (<30 mm, confirmed with abdominal ultrasound) infrarenal aortas. The study was approved by the scientific ethical committees of our institutions, and informed consent was obtained from the patients and the controls for their inclusion in the study.

AAA Tissue and Tissue-Conditioned Medium

Ten AAA tissue samples were collected during surgical repair and dissected into ILT and medial layer. AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol (REflet Sanguin de l'évolutivité des Anévrysmes de l'Aorte abdominale, CCPPRB Paris-Cochin Nos. 2095, 1930, and 1931).[15] All patients gave their written informed consent, and the protocol was approved by a French ethics committee (CCPPRB, Cochin Hospital). AAA thrombus and aortic walls were cut into small pieces (5 mm^2) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (6 mL/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation at 3000*g* for 10 minutes at 20°C.

Human Neutrophils

Neutrophils were obtained from heparinized venous blood by centrifugation in Ficoll-Paque (GE Healthcare) followed by 6% dextran sedimentation of the pellet and hypoosmotic lysis of residual erythrocytes. Neutrophils were resuspended in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, and 30 mmol/L Tris-HCl, pH 8.5), and proteins were precipitated to remove salts and other interfering components by using the 2D Clean Up Kit (GE Healthcare). The resulting proteins were resuspended in lysis buffer. The protein concentration was measured using the RC/DC Protein Assay (Bio-Rad).

PMNs purity was assessed by flow cytometry to ensure that they were not contaminated by other cell types. The analysis of CD16

from PMNs showed a strong fluorescence signal (>90% to 95%) in all samples (Supplemental Figure I).

Differential In-Gel Electrophoresis Experiment and Data Analysis

The 6 paired samples were labeled with CyDye Fluor minimal dyes (GE Healthcare) according to the manufacturer's instructions, and isoelectric focusing and second dimension were performed as described in the supplemental material.

The images were analyzed using the DeCyder software, version 7.0 (GE Healthcare), for spot detection and quantification, intergel matching, and statistics. DeCyder calculates the average abundance of each spot among the 6 gels under study. Statistical significance was assessed for each change in abundance using the Student *t* test ANOVA. We considered spots present in all of the 18 images (3 images per gel) with statistical significance at the 95% confidence level for standardized average spot volume ratios greater than 1.5.

Protein Identification by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

Differentially expressed spots were selected from silver-stained gels for gel excision, automated digestion, and analysis in an Ultraflex matrix-assisted laser desorption ionization (MALDI) tandem time of flight mass spectrometer (Bruker Daltonik) to obtain the corresponding MALDI–mass spectrometry (MS) and MALDI-MS/MS spectra. These MS and MS/MS combined data were used to search a nonredundant protein database (NCBInr; ≈107 entries; National Center for Biotechnology Information, Bethesda, MD) using the Mascot software (Matrix Science). Detailed information is given in the Supplemental Methods.

Western Blot

Cell extracts from PMNs were sonicated and resuspended in lysis buffer, and protein concentration was quantified by Bradford reagent (Bio-Rad). Equal amounts of PMN proteins (20 μg) or an equal volume (10 μL) of AAA tissue–conditioned medium (previously normalized to tissue weight: 6 mL of RPMI per 1g of wet tissue) were used as described in the supplemental material.

Catalase Activity

Similar amounts of PMN extracts were analyzed for catalase activity using a commercial enzymatic assay (K773, Biovision) following the manufacturer's instructions. Catalase units were defined as the amount of enzyme that decomposes 1 μmol H_2O_2 per minute at pH 4.5 at 25°C.

ELISA

We quantified concentrations of catalase and MPO with commercial kits (E92418HU, USCN Life Science and Hycult) following the manufacturers' instructions.

2,7-Dichlorofluorescein Diacetate

Intracellular H_2O_2 levels in PMNs were measured with 2,7-dichlorofluorescein diacetate (Sigma-Aldrich) as described previously.[16] Briefly, 1×10^6 fresh PMNs were loaded with 5 μmol/L 2,7-dichlorofluorescein diacetate in Hanks' buffered salt solution at 37°C for 30 minutes and then washed twice. Fluorescence was evaluated with a microplate reader (GENios Tecan) at 535 nm with an excitatory wavelength of 485 nm for 30 minutes. The reactive oxygen species (ROS) production was expressed as relative fluorescence units per second.

RNA Extraction and Real-Time Quantitative-Polymerase Chain Reaction

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen). One μg of RNA was used to perform the reverse transcribed with High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time polymerase chain reactions were performed on ABI Prism 7500 sequence detection polymerase chain reaction system (Applied Biosystems) according to the manufacturer's protocol using the delta delta Ct method as described.[17] Primers and conditions are described in the supplemental material.

Immunohistochemistry

AAA thrombus samples were fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5-μm sections using Catalase (1:100 Abcam) overnight at 4°C as primary antibody. The peroxidase LSAB + system–horseradish peroxidase kit (Dako) followed by the Histogreen peroxidase substrate (AbCys SA) was used for detection. Sections were then counterstained with Nuclear Fast Red for 5 minutes at room temperature before being mounted using Eukitt medium. Control irrelevant rabbit immunoglobulins (Dako) were applied at the same concentrations as primary antibodies to assess nonspecific staining.

Hydrogen Peroxide Quantification

Quantitative determination of extracellular hydrogen peroxide in ILT conditioned media was performed with a commercial colorimetric kit (907-015, Assay Design), following the manufacturer's instructions.

Statistical Analysis

Results from Western blot, catalase activity, 2,7-dichlorofluorescein diacetate, MPO levels in PMNs, quantitative polymerase chain reaction, and H_2O_2 extracellular levels are expressed as mean±SEM and were analyzed by the Mann-Whitney nonparametric test (small and large AAA and control groups) or Wilcoxon paired test (between thrombus and media supernatants of the same samples). Results from the ELISA are expressed as median (interquartile ranges) and were analyzed by the ANOVA test. A probability value <0.05 was considered statistically significant.

Results

Proteomic Analysis of Circulating PMNs

Proteins from neutrophils isolated from different groups of AAA patients (large AAA [AAA diameter >5 cm] and small AAA [AAA diameter 3–5 cm]) and control subjects (AAA diameter <3 cm) were compared by 2D differential in-gel electrophoresis (Figure 1). DeCyder software provided us a list of differentially expressed spots. After silver staining of the gels, only those visible were excised and trypsin digested. Forty-one of them were finally identified by MALDI MS (Supplemental Table II). Proteins known to be expressed by PMN have been identified (eg, lactoferrin, lipocalin 2). In addition, we found proteins involved in different neutrophil functions, such as cytoskeletal proteins (eg, WDR1), inflammatory processes (eg, LT4H) and survival (eg, coronin). Moreover, a high number of proteins involved in redox balance (eg, cyclophilin, catalase, and thioredoxin reductase) were observed.

Decreased Catalase Levels in Circulating PMNs From AAA Patients

Among the differentially expressed proteins identified by MALDI MS, we have focused on the antioxidant protein catalase because of the importance of oxidative stress in AAA progression. The results derived from proteomic data were validated in a second, independent group of patients and controls by Western blot, confirming the decreased catalase expression in PMNs from patients with large and small AAA relative to control subjects (10.4±0.8 and 10.7±1.2 versus

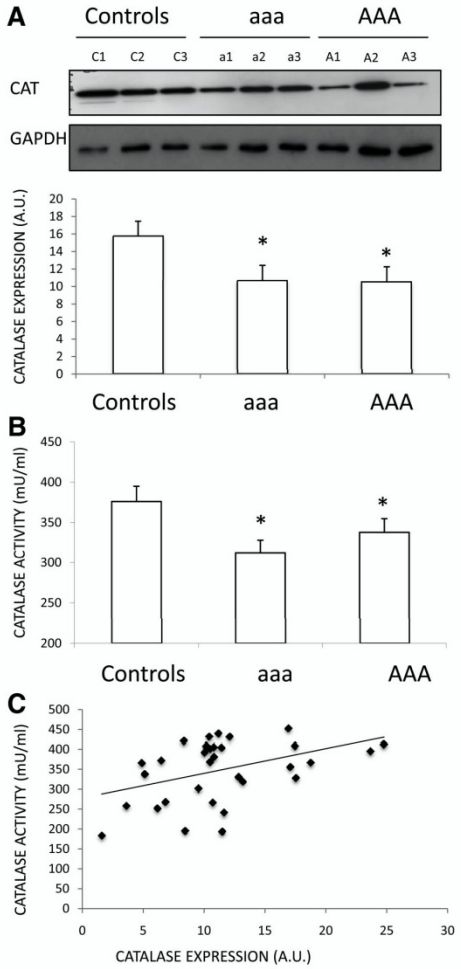


Figure 2. Decreased catalase expression and activity in polymorphonuclear neutrophils (PMNs) from abdominal aortic aneurysm (AAA) patients. **A,** Representative Western blot of catalase and GAPDH in PMN extracts from AAA patients (aaa=AAA <5 cm and AAA=AAA >5 cm) and controls (C). Shown is quantification of catalase expression levels normalized by GAPDH levels in PMNs from AAA patients and controls (n=10, densitometric arbitrary units [A.U.], *P<0.05). **B,** Catalase activity in PMN extracts (n=10, mU/mL, *P<0.05 vs controls). **C,** Correlation between catalase expression and activity in PMN extracts (r=0.4, P<0.05).

15.7±2 arbitrary units, P<0.05, Figure 2A). Similar results were obtained for thioredoxin reductase (10.8±1.5 and 10.6±1.2 versus 14.8±1.7 arbitrary units, P<0.05, not shown). In addition, catalase activity was also assessed in PMNs from patients and controls. As shown in Figure 2B, catalase activity was decreased in PMNs from patients with large and small AAA compared with controls (338±27 and 312±32 versus 376±36 mU/mL, P<0.05). Catalase activity and expression showed a positive correlation (r=0.4, P<0.05, Figure 2C).

Redox Balance of PMNs From AAA Patients and Controls

To address the prooxidant status of neutrophils, we analyzed H_2O_2 and MPO levels from a third additional group of PMNs isolated of 10 AAA patients (AAA >5 cm [n=4] and AAA <5 cm [n=6]) and controls (n=10). PMNs isolated from both large and small AAA patients displayed higher H_2O_2 intracellular levels compared with PMNs from controls (Figure 3A). In addition, PMNs isolated from both large and small AAA patients released higher MPO concentrations compared with PMNs from controls (Figure 3B). In the other hand, baseline catalase mRNA levels were decreased in AAA patients compared with controls (Figure 3C), in agreement with the results obtained at the protein level (Figure 2A). Similar results were obtained for MnSOD mRNA (Figure 3D).

To address whether decreased catalase expression could be associated with the prooxidant conditions that occur in neutrophils during respiratory burst, neutrophils were incubated with PMA, which is known to induce respiratory burst in PMNs. After PMA incubation, increased H_2O_2 and MPO levels were shown in PMNs from controls, reaching levels similar to those of PMNs from patients (Figure 3A and 3B). Interestingly, we found a significant decrease in catalase mRNA levels after PMA incubation, and a similar trend was observed for MnSOD (Figure 3C and 3D).

Decreased Catalase Plasma Levels in AAA Patients

To address whether the redox imbalance observed in circulating PMNs could also occur in plasma of AAA patients, MPO and catalase were assessed in AAA patients and controls. Similar to the results obtained in circulating PMNs, catalase plasma levels were significantly decreased in large and small AAA patients relative to control subjects (111 [63–175] versus 145 [90–208] versus 159 [132–211] U/mL, median [interquartile range], P<0.05 for controls versus small AAA and P<0.001 for controls versus large AAA) (Figure 4A). In the other hand, MPO plasma levels were increased in patients with large and small AAA as compared with controls (65 [46–118] versus 65 [54–92] versus 41 [31–51] ng/mL, P<0.001 for both large and small AAA versus controls; data not shown). Finally, because aortic diameter is a surrogate marker of the growth rate, we studied the correlation between catalase plasma levels and aortic diameter. Interestingly, a significant negative correlation between catalase plasma levels and aortic diameter was found (r=−0.4, P<0.001, Figure 4B).

Catalase in AAA Thrombus and Thrombus-Conditioned Media

Because the luminal layer of ILT of human AAA is enriched in PMNs, we evaluated the levels of catalase in both ILT and ILT-conditioned media. As shown in Figure 5A, the luminal part of the thrombus showed an important staining for catalase, associated with poly-lobed nuclei cells, likely to be

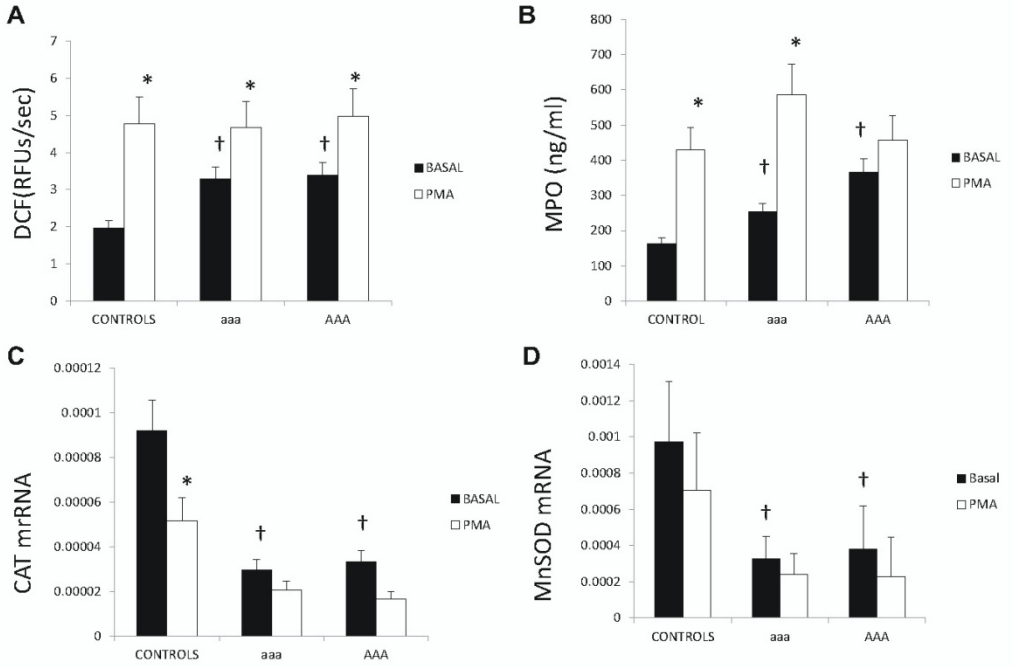


Figure 3. Oxidative status of polymorphonuclear neutrophils (PMNs) from abdominal aortic aneurysm (AAA) patients and controls. PMNs were incubated with vehicle (basal) (black) or with 50 μmol/L phorbol 12-myristate 13-acetate (PMA) (white) for 30 minutes for intracellular H_2O_2 quantification by 2,7-dichlorofluorescein diacetate (DCF) (**A**) or for 4 hours for myeloperoxidase (MPO) determination by ELISA (conditioned media) (**B**) and mRNA expression of catalase (**C**) and manganese superoxide dismutase (MnSOD) (**D**) analysis by quantitative polymerase chain reaction (controls [n=10]; aaa=AAA <5 cm [n=6] and AAA=AAA >5 cm [n=4], *P<0.05 vs basal, †P<0.05 vs controls). RFU, relative fluorescence units.

neutrophils; however, other catalase-positive cells from non-neutrophil origin are observed in AAA tissue (possibly RBCs). Both cellular and diffuse staining was observed, which suggests the presence of catalase in the extracellular compartment. In this respect, catalase levels were increased in the conditioned media of ILT of AAA compared with that of the media layer (15.9±2.7 versus 7.2±1.6 arbitrary units, P<0.05, Figure 5C). Finally, hydrogen peroxide (H_2O_2) levels were also increased in the ILT compared with the media layer (10.4±2.2 versus 5.2±0.7 μmol/L, P<0.05, Figure 5D).

Discussion

PMNs represent the major class of leukocytes. PMNs contribute to main pathological mechanisms of human AAA, such as proteolysis, oxidative stress, and adventitial immune-inflammatory processes.[6–8] The key role of PMNs in the pathogenesis of AAA is supported by recent studies in animal models of AAA.[4,5] Furthermore, therapies modifying PMN content in both human and experimental models of AAA have shown a protective effect on AAA development.[11,12] Because PMNs are key cells in AAA pathophysiology, we have comparatively analyzed circulating PMNs from AAA patients and controls to unveil proteins differentially expressed in pathological conditions, which could provide information about mechanisms involved in AAA evolution. Among identified proteins previously related to AAA, increased lipocalin 2 and cyclophilin have been observed in PMNs of AAA patients in our study. Lipocalin-2 was previously localized in the luminal part of AAA thrombus, associated with matrix metalloproteinase-9.[18] Cyclophilin could participate in different mechanisms involved in vascular remodeling by promoting inflammation and smooth muscle cell proliferation.[19] Moreover, cyclophilin was shown to enhance vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms.[20] These results reinforce the interest and feasibility of analyzing circulating PMNs by proteomic approaches to unveil biomarkers of AAA pathogenesis.

Oxidative stress is the result of an imbalance between antioxidant and prooxidant molecules. Among the proteins identified by proteomic analysis, we showed decreased intracellular expression of antioxidant proteins, such as catalase and thioredoxin reductase, in circulating PMNs from AAA patients compared with controls, whereas cyclophilin was enhanced. Furthermore, we analyzed catalase activity in circulating PMNs, showing a decreased catalase activity of AAA patients compared with controls.

In contrast, we observed that PMNs isolated from AAA patients displayed higher H_2O_2 levels and released higher

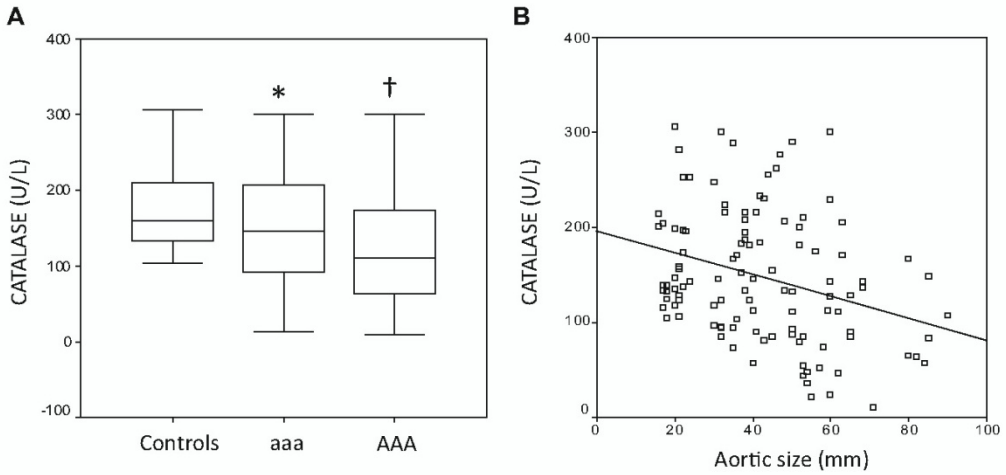


Figure 4. Plasma catalase levels in abdominal aortic aneurysm (AAA) patients. **A,** Catalase levels were significantly increased in plasma from controls (n=34) compared with patients with small AAA (aaa) (n=56) and large AAA (AAA) (n=47) (*$P<0.05$ small AAA vs controls, †$P<0.001$ control vs large AAA). **B,** Correlation between catalase levels and aortic size (Pearson $\rho=-0.4$, $P<0.001$, n=137 subjects). U/L indicates units per liter.

MPO concentrations compared with PMNs from controls, paralleled by a decrease in both catalase and MnSOD mRNA expression. Moreover, to test whether these antioxidant systems could be modified under the conditions of increased oxidative stress associated with respiratory burst, neutrophils were incubated with PMA, known to induce respiratory burst in PMNs. Incubation of PMNs from controls with PMA increased both H_2O_2 and MPO levels, reaching levels similar to those of PMNs from patients, probably associated with the saturation of the prooxidant capacity of these cells. Interestingly, after PMA incubation, a significant decrease in catalase mRNA levels were observed, and a similar trend was obtained for MnSOD, which could suggest a global decrease in antioxidant enzymes in PMNs under respiratory burst conditions. On the whole, our data suggest that a redox imbalance toward increased oxidative stress (increased oxidant species such as H_2O_2 and MPO/decreased antioxidant species such as catalase and MnSOD) was observed in PMNs from AAA patients.

At the systemic level, previous studies have analyzed the circulating levels of different prooxidant molecules in AAA patients.[21] Among them, blood levels of malondialdehyde were significantly increased in AAA patients.[22] Likewise, we have measured MPO in plasma of AAA patients, a well-recognized oxidative stress biomarker of different cardiovascular pathologies,[23,24] showing that MPO plasma levels are increased not only in large AAA,[7] but also in small AAA. In the other hand, catalase plasma levels were significantly decreased in small and large AAA patients relative to control subjects. However, it should be taken into account that patients and controls exhibited differences in risk factors, as well as treatments that could influence oxidative stress. In contrast, small and large AAA patients showed similar risk factors and medications, suggesting that the differences in catalase plasma levels could be associated with the progression of the disease. At the tissue level, superoxide anions, as well as lipid peroxidation products have been assessed in human AAA arterial wall as compared with adjacent nonabdominal segments, showing an increased oxidative stress and associated derived-products in AAA segments.[25] On the other hand, MnSOD activity in human diseased aorta was ≈65% of controls. Furthermore, ruptured AAA tissue also had low SOD activity and protein.[26] However, AAA formation is associated with early increases in SOD expression in an experimental model.[27] In contrast, the beneficial effect of flow loading limiting experimental AAA formation was associated with increased antioxidant gene (hemoxigenase-1) expression in the aorta.[28] In the present study, immunohistochemical analysis showed catalase in human ILT, associated with PMNs. However, other nonnucleated cells, likely RBCs, exhibited a strong immunostaining for catalase. Interestingly, diffuse extracellular staining of catalase was observed in ILT tissue by immunohistochemistry, suggesting its potential release to the extracellular medium. Accordingly, we have shown increased extracellular catalase levels in ILT-conditioned medium, which could be a response to counteract the formation of ROS from extracellular H_2O_2 observed in ILT. In agreement, other authors have shown the presence of catalase in the extracellular medium,[29] and they suggest that catalase secretion could be a response to avoid neutrophil-induced oxidative damage at a local level or to regulate the function of ROS as extracellular signaling molecules. However, high catalase levels in ILT could also be due to cell lysis of both PMNs and RBCs. In this respect, the ILT of AAA is characterized by the presence of several blood cells (among them, PMNs and RBCs) and apoptotic cells, and all of them could contribute to increased oxidative stress. In addition to the release of PMN intracellular content (eg, MPO), the

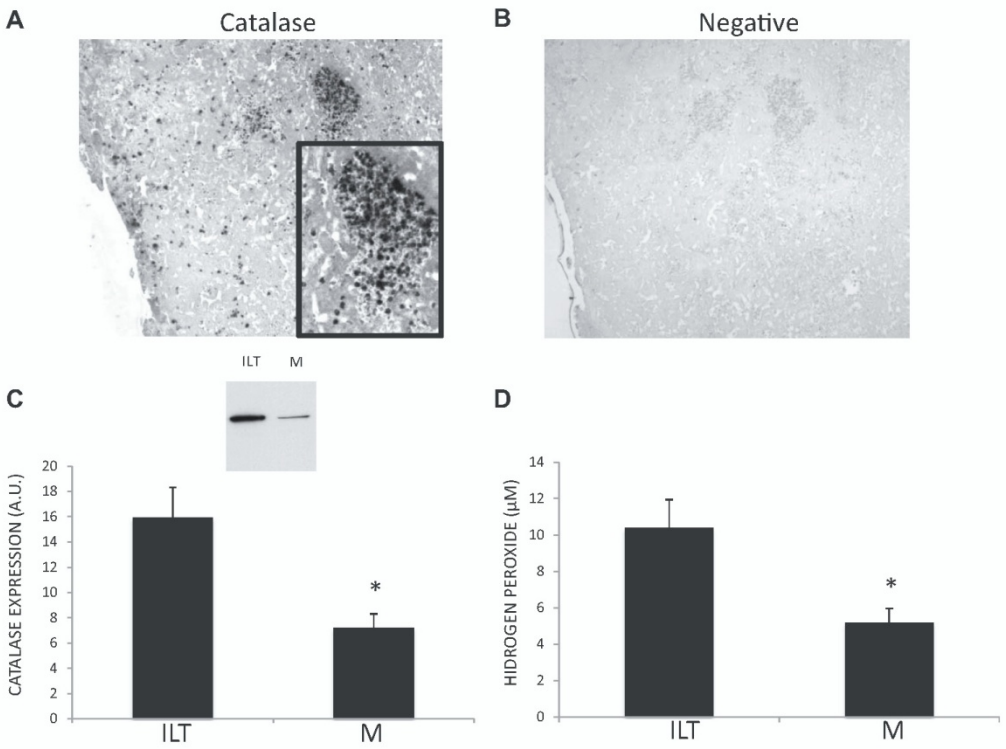


Figure 5. Catalase expression in abdominal aortic aneurysm (AAA) thrombus and thrombus-conditioned medium. **A** and **B,** Immunodetection of catalase in AAA thrombus (**A**) and negative control (**B**) (×10; inset, ×20X). Positivity is shown in green, and nuclei are in red. **C,** Western blot of anticatalase in conditioned medium from intraluminal thrombus (ILT) and medial layer (M) (n=10, **P*<0.001). **D,** Hydrogen peroxide quantification in conditioned media of ILT and healthy media (n=10, *P*<0.05).

trapping of RBCs within the thrombus may lead to hemolysis and subsequent release of hemoglobin, heme, and, finally, prooxidant iron. Among ROS, H_2O_2 is a nonradical, uncharged oxidant that is chemically more stable then other ROS and that can permeate through the vascular wall. In addition, H_2O_2 can accumulate extracellularly in the tissue and survive long enough to induce numerous paracrine functions.[30] H_2O_2 itself is not very reactive; however, the danger of H_2O_2 comes from its rapid conversion to hydroxyl radical by interaction with a range of transition metal ions, of which the most important in vivo is probably iron. Thus, ILT is a privilege site for ROS formation because they can be formed, among other mechanisms, either by MPO-catalyzed or by Fe^{2+}-catalyzed conversion of H_2O_2.[30] On the whole, the imbalance between oxidant species and antioxidant systems in AAA patients, both at the systemic level and the tissue level, further supports the importance of oxidative stress in AAA evolution.

Antioxidant systems are crucial for tissues to detoxify free radical species and protect organisms against oxidative stress. In a previous study, vitamin E attenuated formation of AAA.[31] Importantly, animals treated with vitamin E showed a 44% reduction in the combined end point of fatal and nonfatal aortic rupture. More recently, ROS inhibition has been shown to attenuate aneurysm formation.[32] Among pathological mechanisms potentially modulated by catalase, it was previously shown that ROS/H_2O_2 activates endothelial cells to increase PMN adhesion, and catalase is able to prevent leukocyte accumulation.[33] Moreover, overexpression of catalase suppresses oxidized-low-density lipoprotein–induced aortic smooth muscle cell death[34] and inhibits smooth muscle cell proliferation.[35] In addition, catalase delivery has been successfully used to reduce lipid peroxidation in mice.[36] In these studies, the doses used are lower than its endogenous levels,[37] suggesting that its therapeutic effects could be associated with its functions outside cells.[36] Interestingly, diminished experimental AAA formation by tamoxifen treatment has been associated with increased catalase expression, which was accompanied by decreased PMN infiltration.[38] Furthermore, catalase supplementation inhibited experimental aneurysm formation.[38] Very recently, catalase overexpression in aortic smooth muscle cells prevents pathological mechanical changes underlying AAA formation.[39] On the whole, these studies, including ours suggest a potential protective role of catalase in the mechanisms underlying AAA.

In conclusion, following a proteomic approach to compare circulating PMNs from AAA patients and controls, several proteins have been identified. Among them, we have shown decreased catalase expression and activity in circulating PMNs from AAA patients, paralleled by decreased catalase plasma levels, supporting the main role of oxidative stress in AAA evolution. These results suggest the need for early prevention and treatment of prooxidant factors and for the development of approaches that enhance production or activity of antioxidant enzymes.

Acknowledgments

We thank Patricia Llamas Granda and all the staff of Fundación Jiménez Diaz for helping in the collection of human samples.

Sources of Funding

This work was supported by the European Commission, Fighting Aneurysmal Disease project (FP-7, HEALTH F2-2008-200647), the Spanish Ministerio de Ciencia e Innovacion (SAF2010/21852), Fundacion Ramon Areces, Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035), EUS2008-03565, and Fundacion Pro CNIC.

Disclosures

None.

References

1. Sakalihasan N, Limet R, Defawe OD. Abdominal aortic aneurysm. *Lancet*. 2005;365:1577–1589.
2. Tilson MD. The polymorphonuclear leukocyte and the abdominal aortic aneurysm: a neglected cell type and a neglected disease. *Circulation*. 2005;112:154–156.
3. Baetta R, Corsini A. Role of polymorphonuclear neutrophils in atherosclerosis: current state and future perspectives. *Atherosclerosis*. 2010; 210:1–13.
4. Hannawa KK, Eliason JL, Woodrum DT, Pearce CG, Roelofs KJ, Grigoryants V, Eagleton MJ, Henke PK, Wakefield TW, Myers DD, Stanley JC, Upchurch GR Jr. L-Selectin-mediated neutrophil recruitment in experimental rodent aneurysm formation. Circulation. 2005;112:241–247.
5. Pagano MB, Zhou HF, Ennis TL, Wu X, Lambris JD, Atkinson JP, Thompson RW, Hourcade DE, Pham CT. Complement-dependent neutrophil recruitment is critical for the development of elastase-induced abdominal aortic aneurysm. *Circulation*. 2009;119:1805–1813.
6. Fontaine V, Touat Z, Mtairag el M, Vranckx R, Louedec L, Houard X, Andreassian B, Sebbag U, Palombi T, Jacob MP, Meilhac O, Michel JB. Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus. *Am J Pathol*. 2004;164:2077–2087.
7. Houard X, Touat Z, Ollivier V, Louedec L, Philippe M, Sebbag U, Meilhac O, Rossignol P, Michel JB. Mediators of neutrophil recruitment in human abdominal aortic aneurysms. *Cardiovasc Res*. 2009;82: 532–541.
8. Michel JB, Martin-Ventura JL, Egido J, Sakalihasan N, Treska V, Lindholt J, Allaire E, Thorsteinsdottir U, Cockerill G, Swedenborg J; For the FAD EU consortium. Novel aspects of the pathogenesis of aneurysms of the abdominal aorta in humans. *Cardiovasc Res*. 2011;90:18–27.
9. Thompson RW, Geraghty PJ, Lee JK. Abdominal aortic aneurysms: basic mechanisms and clinical implications. *Curr Probl Surg*. 2002;39: 110–230.
10. Michel JB, Thaunat O, Houard X, Meilhac O, Caligiuri G, Nicoletti A. Topological determinants and consequences of adventitial responses to arterial wall injury. *Arterioscler Thromb Vasc Biol*. 2007;27:1259–1268.
11. Eliason JL, Hannawa KK, Ailawadi G, Sinha I, Ford JW, Deogracias MP, Roelofs KJ, Woodrum DT, Ennis TL, Henke PK, Stanley JC, Thompson RW, Upchurch GR Jr. Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation. *Circulation*. 2005;112:232–240.
12. Lindeman JH, Abdul-Hussien H, van Bockel JH, Wolterbeek R, Kleemann R. Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells. *Circulation*. 2009;119:2209–2216.
13. Golledge J, Tsao PS, Dalman RL, Norman PE. Circulating markers of abdominal aortic aneurysm presence and progression. *Circulation*. 2008; 118:2382–2392.
14. Luerman GC, Uriarte SM, Rane MJ, McLeish KR. Application of proteomics to neutrophil biology. *J Proteomics*. 2010;73:552–561.
15. Caligiuri G, Rossignol P, Julia P, Groyer E, Mouradian D, Urbain D, Misra N, Ollivier V, Sapoval M, Boutouyrie P, Kaveri SV, Nicoletti A, Lafont A. Reduced immunoregulatory CD31+ T cells in patients with atherosclerotic abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. 2006;26:618–623.
16. Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol*. 1994; 55:253–258.
17. Martin-Ventura JL, Madrigal-Matute J, Munoz-Garcia B, Blanco-Colio LM, Van OM, Zalba G, Fortuño A, Gomez-Guerrero C, Ortega L, Ortiz A, Diez J, Egido J. Increased CD74 expression in human atherosclerotic plaques: contribution to inflammatory responses in vascular cells. *Cardiovasc Res*. 2009;83:586–594.
18. Folkesson M, Kazi M, Zhu C, Silveira A, Hemdahl AL, Hamsten A, Hedin U, Swedenborg J, Eriksson P. Presence of NGAL/MMP-9 complexes in human abdominal aortic aneurysms. *Thromb Haemost*. 2007; 98:427–433.
19. Satoh K, Matoba T, Suzuki J, O'Dell MR, Nigro P, Cui Z, Mohan A, Pan S, Li L, Jin ZG, Yan C, Abe J, Berk BC. Cyclophilin A mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation*. 2008;117:3088–3098.
20. Satoh K, Nigro P, Matoba T, O'Dell MR, Cui Z, Shi X, Mohan A, Yan C, Abe J, Illig KA, Berk BC. Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. *Nat Med*. 2009;15:649–656.
21. McCormick ML, Gavrila D, Weintraub NL. Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2007;27:461–469.
22. Papalambros E, Sigala F, Georgopoulos S, Paraskevas KI, Andreadou I, Menenakos X, Sigalas P, Papalambros AL, Vourliotakis G, Giannopoulos A, Bakoyiannis C, Bastounis E. Malondialdehyde as an indicator of oxidative stress during abdominal aortic aneurysm repair. *Angiology*. 2007;58:477–482.
23. Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, Topol EJ, Sprecher DL, Hazen SL. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA*. 2001;286:2136–2142.
24. Martin-Ventura JL, Leclercq A, Blanco-Colio LM, Egido J, Rossignol P, Meilhac O, Michel JB. Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. *Atherosclerosis*. 2007;194:334–341.
25. Miller FJ Jr, Sharp WJ, Fang X, Oberley LW, Oberley TD, Weintraub NL. Oxidative stress in human abdominal aortic aneurysms: a potential mediator of aneurysmal remodeling. *Arterioscler Thromb Vasc Biol*. 2002;22:560–565.
26. Dubick MA, Keen CL, DiSilvestro RA, Eskelson CD, Ireton J, Hunter GC. Antioxidant enzyme activity in human abdominal aortic aneurysmal and occlusive disease. *Proc Soc Exp Biol Med*. 1999;220:39–45.
27. Sinha I, Pearce CG, Cho BS, Hannawa KK, Roelofs KJ, Stanley JC, Henke PK, Upchurch GR Jr. Differential regulation of the superoxide dismutase family in experimental aortic aneurysms and rat aortic explants. *J Surg Res*. 2007;138:156–162.
28. Nakahashi TK, Hoshina K, Tsao PS, Sho E, Sho M, Karwowski JK, Yeh C, Yang RB, Topper JN, Dalman RL. Flow loading induces macrophage antioxidative gene expression in experimental aneurysms. *Arterioscler Thromb Vasc Biol*. 2002;22:2017–2022.
29. Sureda A, Ferrer MD, Tauler P, Maestre I, Aguiló A, Córdova A, Tur JA, Roche E, Pons A. Intense physical activity enhances neutrophil antioxidant enzyme gene expression. Immunocytochemistry evidence for catalase secretion. *Free Radic Res*. 2007;41:874–883.
30. Suvorava T, Kojda G. Reactive oxygen species as cardiovascular mediators: lessons from endothelial-specific protein overexpression mouse models. *Biochim Biophys Acta*. 2009;1787:802–810.
31. Gavrila D, Li WG, McCormick ML, Thomas M, Daugherty A, Cassis LA, Miller FJ Jr, Oberley LW, Dellsperger KC, Weintraub NL. Vitamin E inhibits abdominal aortic aneurysm formation in angiotensin II-infused apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2005; 25:1671–1677.

32. Xiong W, Mactaggart J, Knispel R, Worth J, Zhu Z, Li Y, Sun Y, Baxter BT, Johanning J. Inhibition of reactive oxygen species attenuates aneurysm formation in a murine model. *Atherosclerosis*. 2009;202:128–134.
33. Gasic AC, McGuire G, Krater S, Farhood AI, Goldstein MA, Smith CW, Entman ML, Taylor AA. Hydrogen peroxide pretreatment of perfused canine vessels induces ICAM-1 and CD18-dependent neutrophil adherence. *Circulation*. 1991;84:2154–2166.
34. Guo ZM, Van Remmen H, Yang H, Chen XL, Mele J, Vijg J, Epstein CJ, Ho YS, Richardson A. Changes in expression of antioxidant enzymes affect cell-mediated LDL oxidation and oxLDL-induced apoptosis in mouse aorta cells. *Arterioscler Thromb Vasc Biol*. 2001;21:1131–1138.
35. Shi MJ, Yang H, Motley ED, Guo ZM. Overexpression of Cu/Znsuperoxide dismutase and/or catalase in mice inhibits aorta smooth muscle cell proliferation. *Am J Hypertens*. 2004;17:450–456.
36. Nishikawa M, Hashida M, Takakura Y. Catalase delivery for inhibiting ROS-mediated tissue injury and tumor metastasis. *Adv Drug Deliv Rev*. 2009;61:319–326.
37. Nishikawa M, Tamada A, Kumai H, Yamashita F, Hashida M. Inhibition of experimental pulmonary metastasis by controlling biodistribution of catalase in mice. *Int J Cancer*. 2002;99:474–479.
38. Grigoryants V, Hannawa KK, Pearce CG, Sinha I, Roelofs KJ, Ailawadi G, Deatrick KB, Woodrum DT, Cho BS, Henke PK, Stanley JC, Eagleton MJ, Upchurch GR. Tamoxifen up-regulates catalase production, inhibits vessel wall neutrophil infiltration, and attenuates development of experimental abdominal aortic aneurysms. *J Vasc Surg*. 2005;41:108–114.
39. Maiellaro-Rafferty K, Weiss D, Joseph G, Wan W, Gleason RL, Taylor WR. Catalase overexpression in aortic smooth muscle prevents pathological mechanical changes underlying abdominal aortic aneurysm formation. *Am J Physiol Heart Circ Physiol*. 2011;301:H355–H362.

SUPPLEMENT MATERIAL

METHODS

DIGE protein labeling, 2DE and image acquisition and DIGE data analysis

Samples were labeled with CyDye Fluor minimal dyes (GE Healthcare) according to manufacturer´s instructions. Briefly, 50µg of protein extracts were mixed with 400pmol of the N-hydroxysuccinimide esters of Cy3 or Cy5 fluorescent cyanine dyes on ice in the dark for 30 min. An equal amount of each sample included in the experimental sample set was combined to create the internal standard. The internal standard was labeled with Cy2. The labeling reactions were stopped with 1μL of 10mM lysine on ice in the dark for 10min.

The six-paired samples of Cy3 and Cy5-labeled proteins were mixed with 50µg of Cy2-labeled internal standard. The mixtures were diluted in Rehydration Buffer (7M urea, 2M thiourea, 4% CHAPS, 0.8% IPG Buffer 3-11NL and bromophenol blue) containing 50mM DTT, and resolved on 24cm IPG strips pH3–11 non-linear gradient IPG strips. The samples were applied by cup loading to the previously rehydrated IPG strips with 450µL of the mentioned Rehydration Buffer containing 97mM DeStreak reagent (GE Healthcare). The isoelectric focusing (IEF) was performed using an IPGphor II IEF system (GE Healthcare) until a total of 42kVh following a stepwise voltage increase: 300V for 3h, linear gradient to 1000V in 4h, linear gradient to 8000V in 2h and 8000V until the steady state was reached.IEF strips were then equilibrated in buffer containing 6M urea, 30% glycerol, 2% SDSand 30mM Tris-HCl and trace amounts of bromophenol blue for 15 minutes with addition of 1% DTT. Finally, strips were incubated with the same buffer containing 4% iodoacetamide instead of DTT for 15 additional minutes. Second dimension was performed on 12% polyacrylamide gels at 17W/gel using an Ettan Dalt Six device (GE Healthcare). Gels

were scanned on a Typhoon 9400 (GE Healthcare) and Cy2-, Cy3-, and Cy5-labeled images of each gel were acquired at excitation/emission wavelength values of 488/520, 523/580 and 633/670 nm respectively. Finally, gels were fixed in 12% methanol and 7% acetic acid, and silver stained using a commercial kit (GE Healthcare).

The images were analyzed using the DeCyder version 7.0 software (GE Healthcare) for spot detection and quantification, inter-gel matching and statistics. DeCyder calculates the average abundance of each spot among the six gels under study. Statistical significance was assessed for each change in abundance using Student´s t-test ANOVA analysis. We considered spots present in all of the 18 images (three images per gel) with statistical significance at 95% confidence level for standardized average spot volume ratios over 1.5.

In-Gel Trypsin Digestion

Protein spots from silver-stained gels were manually excised from gels, and transferred to pierced V-bottom 96-well polypropylene microplates (Bruker Daltonik). Samples were digested automatically using a Proteineer DP protein digestion station (Bruker Daltonik). The gel pieces were submitted to reduction with 10 mM DTT (GE Healthcare) in 50mM ammonium bicarbonate (99.5% purity; Sigma Chemical) and alkylation with 55mM iodoacetamide (Sigma Chemical) in 50mM ammonium bicarbonate. Gel pieces were then washed with 50mM ammonium bicarbonate and acetonitrile (gradient grade; Merck) and dried with a nitrogen stream. The samples were digested with trypsin 8ng/µL at 37°C for 8h (sequencing grade; Promega) 50mM ammonium bicarbonate was added to the dry gel pieces. After digestion, the peptides were extracted with 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical).

MALDI Mass spectrometry

Dried samples were dissolved in 0.2g/l α-cyano-4-hydroxycinnamic acid (Bruker Daltonik) in 50% aqueous acetonitrile and 0.2% trifluoroacetic acid (99.5% purity; Sigma Chemical). This solution was deposited onto a 600μm AnchorChip prestructured MALDI probe (Bruker Daltonik) and allowed to dry at room temperature. Samples were automatically analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) with an automated analysis loop controlled by the flexControl 2.2 software (Bruker Daltonik). In a first step, MALDI-MS spectra were acquired by averaging 400 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in a mass range from 800 to 3500 Da. Internal calibration of MALDI-MS mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105. In a second step, precursor ions showing in the MALDI-MS mass spectrum were subject to fragment ion analysis in the tandem (MS/MS) mode to average 1200 spectra. For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. Automated analysis of mass data was performed using the flexAnalysis 2.2 software (Bruker Daltonik). No smoothing or any further spectral processing was applied. MALDI-MS and MS/MS spectra were manually inspected in detail and reacquired, recalibrated and/or relabelled using the aforementioned programs and homemade software when necessary.

MALDI-MS Database searching

MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search a nonredundant protein database (NCBInr 20091015, ~ 10^7 entries, National Center for Biotechnology Information, Bethesda US), using the Mascot software (Matrix Science, London, UK; http://www.matrixscience.com).

Other relevant search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); allow up to 1 missed cleavage; peptide tolerance ±20 ppm; MS/MS tolerance ±0.5 Da. Protein scores greater than 82 were considered significant ($p<0.05$).

Western-blot

Cell extracts from PMNs were sonicated, resuspended in lysis buffer and protein concentration was quantified by Bradford reagent (BioRad). Equal amount of PMN proteins (20μg) or equal volume (10μl) of AAA tissue conditioned-media (previously normalized to tissue weight: 6mL RPMI/1g of wet tissue) was runned on denaturing SDS/12% (w/v) polyacrylamide gels. Proteins were then blotted onto PVDF (Immobilion-P; Millipore) membranes and the blots were blocked with 10% (w/v) non-fat dry milk in TBST (0.01M Tris (pH 7.7), 0.1M NaCl and 0.1% Tween 20). The membranes were incubated with monoclonal antibodies against either Catalase (1:1000 abcam) or GADPH (1:5000 Santa Cruz). After, they were incubated with HRP (horseradish peroxidase)-conjugated anti-(rabbit or mouse IgG) antibodies at a dilution of 1:2500. The proteins were then detected by enhanced chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare) and evaluated by densitometry (Quantity One; BioRad Laboratories). Pre-stained protein markers (PageRuler™ Prestained Protein Ladder; Fermentas) were used for molecular mass determinations

Real-time PCR

Quantification of Catalase and MnSOD mRNA levels were done by amplification of cDNA using SYBR® Green. The primer sequences were as follows: Catalase (NM_001752.3; predicted size 210 bp) sense: 5′- TTAATCCATTCGATCTCACC -3′, and antisense: 5′- GGCGGTGAGTGTCAGGATAG -3′; MnSOD (NM_000636.2,

predicted size 54 bp) sense: 5′- CACTCGTGGCTGTGGTGGCT -3′, and antisense: 5′- GCTGATGCCGCCGATCTGCT -3′; 18S (NR_003286.2, predicted size 125 bp) sense: 5′- CCGTCGTAGTTCCGACCATAA -3′, and antisense 5′- CAGCTTTGCAACCATACTCCC -3′. Expression levels are given as ratio to housekeeping gene 18S and data is expressed as ΔCt. The relative quantification was done using the comparative CT method and expressed as arbitrary units.

Table I online. Characteristics of the patients included in the study.

	Small aaa patients (*n=56*)	Large AAA patients (*n=47*)
Age (years)	72±8	71±8
Sex (Male/Female)	54 (96%) / 2 (4%)	45 (96%) / 2 (4%)
Active smoking	18/56 (32%)	15/47 (32%)
Hypertension	38/56 (68%)	23/47 (49%)
Diabetes mellitus	14/56 (25%)	8/47 (17%)
Hypercholesterolemia	32/56 (57%)	29/47 (62%)
Cardiac disease	13/56 (23%)	7/47 (15%)
Chronic Obstructive Pulmonary Disease	10/56 (18%)	9/47 (19%)
Chronic renal failure	0/56 (0%)	4/47 (9%)

TABLE II online. *Proteins altered in PMNs from AAA patients and control subjects found by 2D-DIGE/MS*

Spot *	DeCyder †						Protein description §	Accession code §	Mascot #						
	C vs aaa		C vs AAA		aaa vs AAA				Score	Expected	Ions score	MW(KDa)/ pI**	Matched ††	Unmatched ††	Cov.††
	Av Ratio	p-value	Av Ratio	p-value	Av Ratio	p-value									
1	--	--	-1.2	8.2E-02	-1.35	3.1E-02	Lactoferrin	gi|28948741	108	1.60E-04	NA	77.93/8.47	8	4	12
2	1.68	4.2E-02	3.6	6.3E-02	--	--	Guanine nucleotide-binding protein G(i)	gi|23398623	214	4.00E-15	97	39.10/5.27	6	0	14
3	1.52	4.5E-02	--	--	--	--	Actin cytoplasmic 1	gi|15277503	172	1.40E-12	93	40.54/5.55	4	0	16
4	-1.34	7.4E-02	1.58	4.7E-02	2.12	4.3E-02	Actin cytoplasmic 2	gi|178045	162	1.40E-11	102	26.15/5.65	3	0	20
5	--	--	1.41	6.3E-02	--	--	glyoxalase domain containing 4	gi|217330598	191	8.00E-13	NA	33.55/5.4	11	3	41
6	1.86	5.4E-02	2.6	6.7E-02	--	--	Actin cytoplasmic 1	gi|15277503	535	7.10E-49	346	40.54/5.55	11	3	35
7	1.88	3.4E-02	2.98	2.1E-02	--	--	Actin cytoplasmic 1	gi|15277503	139	2.80E-09	NA	40.54/5.55	7	0	25
8	--	--	--	--	-1.83	4.2E-02	Copine-3	gi|4503015	172	6.30E-11	68	60.95/5.6	6	0	12
9	--	--	-128	1.1E-02	--	--	ubiquitin-activating enzyme E1	gi|23510338	126	2.50E-06	NA	118.86/5.49	9	1	10
10	--	--	1.26	3.3E-02	--	--	alpha-actinin 1	gi|54304187	79	2.60E-03	54	10.19/5.41	1	0	13
11	1.48	0.03	1.66	6.7E-02	--	--	Major vault protein	gi|1097308	203	5.00E-14	58	100.14/5.34	9	0	15
12	1.52	1.4E-02	--	--	--	--	ARP3 actin-related protein 3 homolog B	gi|5031573	149	1.30E-08	NA	47.80/5.61	9	4	23
13	--	--	-1.69	0.03	-1.48	9.2E-02	Nucleoside diphosphate kinase A	gi|35068	136	5.70E-09	62	20.74/7.07	4	2	26
14	-1.31	7.8E-02	-2.14	5.6E-03	-1.63	2.3E-02	gelsolin	gi|55960301	135	7.10E-09	54	29.11/7.71	4	0	13
15	1.92	3.6E-02	2.41	7.1E-02	--	--	Leukotriene A4 Hydrolase	gi|51247429	231	8.00E-17	49	69.65/5.73	11	1	25
16	--	--	1.42	3.3E-02	1.46	0.02	Thioredoxin reductase 1, cytoplasmic	gi|3820535	101	1.80E-05	NA	55.32/6.36	7	4	16
17	1.43	3.1E-02	1.93	9.9E-02	--	--	Coronin-1A	gi|5902134	257	4.50E-21	119	51.39/6.25	9	4	18
18	1.54	4.3E-02	--	--	--	--	Actin-related protein 3B	gi|5031571	233	1.10E-18	115	45.02/6.3	8	6	29
19	--	--	--	--	1.28	3.9E-02	Proteasome subunit alpha type-7	gi|4506179	265	7.10E-22	137	29.62/6.15	7	2	36
20	-2.29	2.3E-02	--	--	NS		phosphoglycerate mutase 1	gi|114326546	141	8.00E-08	NA	28.93/6.67	7	4	25
21	--	--	-1.76	2.8E-02	-1.51	1.6E-02	phosphoglycerate mutase 1	gi|4505753	162	1.40E-11	NA	28.90/6.67	10	1	51
22	--	--	--	--	2.12	3.3E-02	phosphoglycerate mutase 1	gi|114326546	141	8.00E-08	NA	28.93/6.67	7	1	38
23	--	--	-1.43	8.4E-02	--	--	Rho GDP-dissociation inhibitor 1	gi|4757768	263	1.10E-21	163	23.25/5.02	6	2	25
24	1.58	0.03	--	--	--	--	transaldolase	gi|5803187	104	4.00E-04	NA	37.69/6.36	7	2	24
25	--	--	1.61	0.03	1.62	8.4E-02	adenosine kinase	gi|32484975	107	2.00E-04	NA	40.92/6.24	6	0	12
26	--	--	--	--	1.38	4.3E-02	Coronin-1A	gi|5902134	206	2.50E-14	51	51.68/6.25	12	7	20
27	--	--	3.48	3.80E-03	--	--	catalase	gi|4557014	440	1.00E-37	175	59.95/6.9	17	5	42
28	1.62	5.6E-02	2.27	9.5E-02	--	--	esterase D/formylglutathione hydrolase	gi|33413400	256	2.50E-19	123	31.96/6.54	8	5	30
29	--	--	1.69	3.4E-02	--	--	phosphoglycerate mutase 1	gi|4505753	400	2.30E-35	189	28.90/6.67	12	7	56
30	--	--	-1.86	2.8E-02	--	--	adenylyl cyclase-associated protein	gi|5453595	146	5.70E-10	69	51.93/8.07	5	0	10
31	--	--	1.19	9.1E-03	1.36	5.7E-02	Ras-related protein Rab-7a	gi|34147513	214	9.00E-17	87	23.76/6.4	6	0	40
32	--	--	2.04	2.9E-03	2.14	6.3E-02	adenylyl cyclase-associated protein	gi|5453595	193	1.10E-14	96	51.93/8.07	7	3	21
33	--	--	2.69	8.4E-03	3.01	5.02E-03	DNA-(apurinic or apyrimidinic site) lyase	gi|16375501	172	1.40E-12	52	35.93/8.33	7	2	30
34	--	--	3.46	3.1E-02	5.47	3.2E-02	gelsolin	gi|38044288	380	2.30E-33	284	80.88/5.58	8	7	11
35	--	--	3.39	2.4E-02	4.37	0.02	gelsolin	gi|38044288	176	5.70E-13	104	80.88/5.58	5	1	6
36	2.12	5.6E-02	3.1	2.9E-02	--	--	carbonic anhydrase I	gi|4502517	124	9.00E-08	NA	28.91/6.59	6	1	38
37	--	--	-1.73	2.6E-02	-1.39	2.3E-02	Human Seminal Lactoferrin	gi|28948741	1220	1.00E-115	647	77.93/8.47	36	2	61
38	2.06	4.1E-02	--	--	--	--	phosphoglycerate kinase 1	gi|4505763	178	3.60E-13	84	44.99/8.3	6	1	20
39	--	--	2.69	8.4E-03	3.01	5.03E-03	adenylyl cyclase-associated protein	gi|5453595	170	2.30E-12	119	51.93/8.07	3	1	11
40	-1.27	5.56E-03	--	--	--	--	lipocalin 2	gi|55961101	146	5.70E-10	67	22.95/8.66	4	1	22
41	-1.35	9.9E-02	--	--	--	--	cyclophilin	gi|181250	190	2.30E-14	97	22.65/9.33	5	0	23

* Spot number according to Figure 1

†Average volume ratio and p-values from t-test as quantified by DeCyder software (--: Spots without statistical significance; C, Control; aaa, small AAA; AAA, large AAA; Higher protein expression levels is indicated by positive average ratios)

§ Protein ID and accession number according to NCBI database

Mascot score, expectation value and ions score (NA: Not applicable, that applies to proteins identified just with their corresponding Protein Mass Fingerprint. Ions score applies to peptide MS/MS fragmentation spectra)

** Theoretical protein molecular weight (MW) and isoelectric point (pI)

†† Number of peptides matching the protein sequence, number of unmatched peptides and percentage of protein sequence coverage (Cov).

Arteriosclerosis, Thrombosis, and Vascular Biology
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®
Learn and Live℠

Identification of Peroxiredoxin-1 as a Novel Biomarker of Abdominal Aortic Aneurysm

Roxana Martinez-Pinna, Priscila Ramos-Mozo, Julio Madrigal-Matute, Luis M. Blanco-Colio, Juan A. Lopez, Enrique Calvo, Emilio Camafeita, Jes S. Lindholt, Olivier Meilhac, Sandrine Delbosc, Jean-Baptiste Michel, Melina Vega de Ceniga, Jesus Egido and Jose L. Martin-Ventura

Arterioscler Thromb Vasc Biol 2011, 31:935-943: originally published online January 27, 2011
doi: 10.1161/ATVBAHA.110.214429
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online ISSN: 1524-4636

Clinical and Population Studies

Identification of Peroxiredoxin-1 as a Novel Biomarker of Abdominal Aortic Aneurysm

Roxana Martinez-Pinna, Priscila Ramos-Mozo, Julio Madrigal-Matute, Luis M. Blanco-Colio, Juan A. Lopez, Enrique Calvo, Emilio Camafeita, Jes S. Lindholt, Olivier Meilhac, Sandrine Delbosc, Jean-Baptiste Michel, Melina Vega de Ceniga, Jesus Egido, Jose L. Martin-Ventura

Objective—In the search of novel biomarkers of abdominal aortic aneurysm (AAA) progression, proteins released by intraluminal thrombus (ILT) were analyzed by a differential proteomic approach.

Methods and Results—Different layers (luminal/abluminal) of the ILT of AAA were incubated, and the proteins released were analyzed by 2-dimensional difference in-gel electrophoresis. Several differentially expressed proteins involved in main AAA pathological mechanisms (proteolysis, oxidative stress, and thrombosis) were identified by mass spectrometry. Among the proteins identified, peroxiredoxin-1 (PRX-1) was more released by the luminal layer compared with the abluminal layer of the ILT, which was further validated by Western blot, ELISA, and immunohistochemistry. We demonstrated increased PRX-1 serum levels in AAA patients compared with healthy subjects and also positive correlation among PRX-1 and AAA diameter, plasmin-antiplasmin, and myeloperoxidase levels. Finally, a prospective study revealed a positive correlation between PRX-1 serum levels and AAA expansion rate. Moreover, the combination of PRX-1 and AAA size had significantly additive value in predicting growth.

Conclusion—Several proteins associated with AAA pathogenesis have been identified by a proteomic approach in ILT-conditioned medium. Among them, PRX-1 serum levels are increased in AAA patients and correlate with AAA size and growth rate, suggesting the potential use of PRX-1 as a biomarker for AAA evolution. (***Arterioscler Thromb Vasc Biol.* 2011;31:935-943.**)

Key Words: aneurysms ■ antioxidants ■ proteomics

Abdominal aortic aneurysm (AAA) is an important health problem, which occurs in up to 9% of adults older than 65 years of age. The incidence of asymptomatic and ruptured AAA has increased during recent decades, causing ≈1% to 2% of male deaths in Western countries.[1] Because AAAs are usually asymptomatic before rupture, the present clinical challenges are to diagnose AAA at an early stage and to decipher the biological mechanisms leading to progressive dilatation and finally rupture, to develop new diagnostic and therapeutic approaches. Identification of biomarkers could help to target both objectives.

Previous studies have identified AAA biomarkers by studying the levels of different molecules potentially related to AAA pathological mechanisms.[2,3] A different noncandidate biomarker strategy using a set of modern high-throughput technologies, including proteomics, will offer new opportunities to gain a deeper insight into disease processes, including their molecular mechanisms, the risk factors involved, and the analysis of disease progression.[4,5] We have previously reported a differential proteomic approach to identify new atherothrombosis biomarkers released by the arterial wall into plasma using normal and pathological arteries in culture.[6,7] The presence of an intraluminal thrombus (ILT) is a main feature of AAA, and recent data suggest that the biological activities of ILT play a major role in AAA development in humans.[8] In this study, different layers (luminal/abluminal) from the ILT of human AAA patients were incubated in protein-free medium, and the released proteins were analyzed by a gel-based (2-dimensional difference in-gel electrophoresis [2D-DIGE]) proteomic approach followed by protein identification by mass spectrometry (MS). We focused on peroxiredoxin-1 (PRX-1) in view of its potential role in modulating oxidative stress and thus validated its differential expression by Western blot and ELISA in the conditioned medium. Finally, the suitability of PRX-1 as a

Received on: August 9, 2010; final version accepted on: January 12, 2011.

From the Vascular Research Laboratory, Instituto de Investigación Sanitaria-Fundación Jiménez Diaz-Autonoma University, Madrid, Spain (R.M.-P., P.R.-M., J.M.-M., L.M.B.-C., J.E., J.L.M.-V.); Unidad de Proteómica, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain (J.A.L., E. Calvo, E. Camafeita); Vascular Research Unit, Viborg Hospital, Viborg, Denmark (J.S.L.); Institut National de la Santé et de la Recherche Médicale U698, Paris, France (O.M., S.D., J.-B.M.); Université Denis Diderot, Paris, France (O.M., S.D., J.-B.M.); Assistance Publique Hopitaux de Paris, Centre Hospitalier Universitaire, Bichat-Claude Bernard, Paris, France (O.M., S.D., J.-B.M.); Vascular Surgery, Hospital Galdakao, Bilbao, Spain (M.V.d.C.).

Correspondence to Jose Luis Martin-Ventura, PhD, Vascular Research Laboratory, Fundacion Jimenez Diaz, Autonoma University, Av Reyes Catolicos 2, 28040, Madrid, Spain. E-mail jlmartin@fjd.es

Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.110.214429**

biomarker of AAA presence and progression was assessed in sera from 2 different population studies by ELISA.

Materials and Methods

AAA Tissue and Tissue-Conditioned Media

Ten human AAA thrombus samples were collected during surgical repair and dissected into luminal and abluminal parts (at the interfaces with circulating blood and with the remaining media, respectively). AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol (Reflet sanguin de l'évolutivité des anévrysmes de l'aorte abdominale; Comités Consultatifs de Protection des Personnes dans la Recherche Biomédicale Paris-Cochin numbers 2095, 1930, and 1931).[9] All patients gave their informed written consent, and the protocol was approved by a French ethics committee (Comités Consultatifs de Protection des Personnes dans la Recherche Biomédicale, Cochin Hospital). Luminal and abluminal layers of AAA thrombus were cut into small pieces (5 mm^3) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (6 mL/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation at 3,000*g* for 10 minutes at 20°C. The protein concentration of each conditioned medium was measured using the Bradford assay (Bio-Rad).

Sample Preparation and DIGE Protein Labeling

Supernatants from luminal and abluminal layers of ILT human samples from 4 individual patients were isolated, and the proteins were precipitated using the 2D clean-up kit (GE Healthcare) and resuspended in 30 mmol/L Tris-HCl, pH 8.5, 7 mol/L urea, 2 mol/L thiourea, and 40 g/L CHAPS. Protein concentration was measured by RC-DC protein assay kit (Bio-Rad).

The comparison between the proteins released by luminal and abluminal layers was performed by 2D-DIGE analyses across 4 gels, using the same pooled-sample internal standard, the equimolecular mixture of all the samples, in all gels. The samples were labeled according to manufacturer's instructions for minimal labeling, using 400 pmol of dye reagent for 50 μg of protein extract. Individual samples were labeled with Cy3 or Cy5 dyes using dye switching, and the internal standard was always Cy2 labeled. The labeling reaction was performed on ice for 30 minutes in darkness and quenched by 1 μL of lysine (10 mmol/L) for 10 minutes.

2D Electrophoresis and Image Acquisition

The 4-paired samples of Cy3- and Cy5-labeled proteins were mixed with 50 μg of Cy2-labeled internal standard. The mixtures were diluted in rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 40 g/L CHAPS, 0.8% IPG buffer 3-11NL, and bromphenol blue) containing 50 mmol/L immobilized pH gradient and resolved on 24-cm pH 3–11 nonlinear gradient IPG strips (GE Healthcare). The samples were applied by cup loading to the previously rehydrated IPG Strips with 450 μL of the aforementioned rehydration buffer containing 97 mmol/L DeStreak (GE Healthcare).

The isoelectric focusing was carried out in an IPGphor II isoelectric focusing system (GE Healthcare) until a total of 42 kVh was reached. After isoelectric focusing, strips were equilibrated in buffer containing 6 mol/L urea, 400 mmol/L glycerol, 7 mmol/L SDS, and bromphenol blue, for 15 minutes with addition of 6.5 mmol/L dithiothreitol, and then without dithiothreitol but with the same buffer supplemented with 21.6 mmol/L iodoacetamide for an additional 15 minutes. SDS-PAGE was carried out on 12% polyacrylamide gels at 2 W/gel.

The differentially labeled coresolved proteins in each gel were acquired with a Typhoon 9400 laser scanner (GE Healthcare), and Cy2-, Cy3-, and Cy5-labeled images of each gel were acquired at excitation/emission wavelength values of 488/520, 523/580, and 633/670 nm, respectively. Finally, gels were fixed in 12% methanol and 7% acetic acid and silver stained using the Plus One silver staining kit (GE Healthcare).

DIGE Data Analysis

The images were analyzed using the DeCyder version 6.5 software (GE Healthcare) for spot detection and quantification and intergel matching and statistics. DeCyder calculates the average abundance for each spot among the 4 gels under study. Statistical significance was assessed for each change in abundance using the paired Student *t* test analysis. We considered spots present in all of the 12 images (Cy2-, Cy3-, and Cy5-labeled images of each gel) with statistical significance at 95% confidence level for standardized average spot volume ratios greater than 1.5.

Protein Identification by Matrix-Assisted Laser Desorption Ionization-MS

Differentially expressed spots were selected from silver-stained gels for gel excision, automated digestion,[10] and analysis in an Ultraflex matrix-assisted laser desorption ionization (MALDI) time-of-flight/time-of-flight mass spectrometer (Bruker Daltonik) to obtain the corresponding MALDI-MS and MALDI-MS/MS spectra as described.[11,12] These MS and MS/MS combined data were used to search a nonredundant protein database (NCBInr; $\approx 10^7$ entries; National Center for Biotechnology Information, Bethesda, MD) using the Mascot software (Matrix Science).[13] Detailed information is included the Supplemental Methods, available online at http://atvb.ahajournals.org.

Western Blot

Equal amounts (50 μg of protein) of conditioned medium were loaded onto 12.5% polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes. Then they were blocked with 7% milk powder in TBS-T for 1 hour and incubated overnight at 4°C at 1:500 with anti-peroxiredoxin antibody (Santa Cruz Biotechnology, goat polyclonal sc-23969). Then, the membranes were washed with TBS-T and incubated with anti-goat antibody (1:2000) for 1 hour at room temperature. After 4 washes, the signal was detected using the ECL chemiluminiscence kit (GE Healthcare).

ELISA

The soluble concentration of PRX-1 in both conditioned media and serum was quantified with a commercial kit following the manufacturer's instructions (AbFrontier). The interassay and intraassay variability values were 9% and 6%, respectively.

Immunohistochemistry

AAA thrombus samples were fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5-μm sections, using rabbit anti-peroxiredoxin 1 (ab-15571) at 1:100 overnight at 4°C as the primary antibody. The peroxidase LSAB+ system horseradish peroxidase kit (Dako), followed by Histogreen peroxidase substrate (AbCys SA), was used for detection. Sections were then counterstained with Nuclear Fast Red for 5 minutes at room temperature before being mounted using Eukitt medium. Control irrelevant rabbit immunoglobulins (Dako) were applied at the same concentrations as primary antibodies to assess nonspecific staining.

Red Blood Cell In Vitro Experiments

Blood was collected in EDTA-tubes from 6 healthy volunteers. The blood sample was centrifuged at 2500 rpm for 15 minutes to eliminate plasma, and then blood cells were diluted 1:1 in PBS (154 mmol/L NaCl, 10 mmol/L phosphate buffer, pH 7.4) and were separated by centrifugation in Ficoll-Paque (GE Healthcare). Finally, red blood cells (RBCs) and leukocytes were separated by 6% dextran sedimentation of the pellet. Erythrocytes were incubated with lipopolysaccharide (0.1 and 1 ng/mL) and H_2O_2 (5 and 500 μmol/L) for 30 minutes. The lysis of RBCs was achieved by hypotonic shock, using repetitive washes with distilled water, and finally by adding NaCl (3.5%). Membrane fractions were collected after centrifugation at 12 000 rpm for 10 minutes.

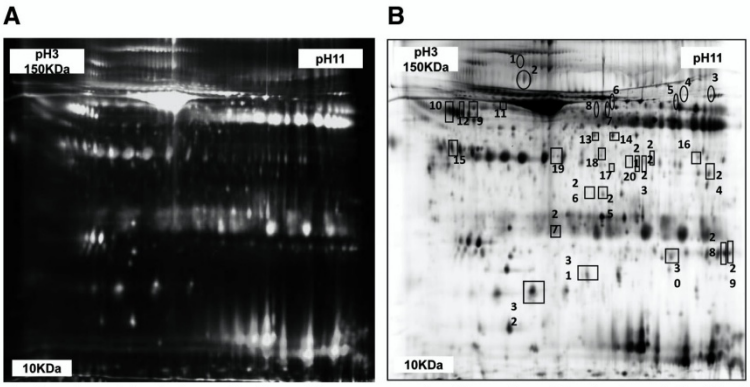


Figure 1. DIGE of AAA-conditioned media. Luminal and abluminal layers from ILT of 4 AAA patients were incubated in protein-free medium. Secreted proteins were labeled with the corresponding CyDye reagents, mixed, resolved on 4 independent DIGE gels, and imaged (red, Cy3; green, Cy5; blue, Cy2) and analyzed with DeCyder software. A, A representative gel image from the 3-plex DIGE experiment is shown. Proteins were resolved in the 3 to 11 (nonlinear) pH range in the first dimension and on 12% polyacrylamide gels in the second dimension. B, Spots showing statistically significant regulation between the 2 conditions were excised from silver-stained gels and identified by MALDI-MS. Identified spots are numbered as listed in the Table.

Hydrogen Peroxide Quantification

Quantitative determination of hydrogen peroxide was performed with a commercial colorimetric kit (907-015, Assay Design) following the manufacturer's instructions.

AAA Patient Sera

Spanish Study

The study was approved by the Spanish center's research and ethics committees, and informed consent from the patients and the controls for their inclusion in the study was obtained. Eighty-three consecutive patients with an asymptomatic infrarenal AAA were recruited, and for each, the AAA size at the time of blood sample collection was registered (Supplemental Table I). We excluded patients with symptomatic or inflammatory AAA, multiple synchronic aneurysms (thoracic, femoral, popliteal), and AAA with a location other than infrarenal. We also excluded patients with active inflammatory or acute infectious processes, surgical procedures or major trauma in the previous 60 days, and chronic antiinflammatory or immunosupressive medication.

Thirty-three controls were recruited from a screening program, which is currently being performed among the population in the area under our care.[14] They were randomly selected from the screened individuals with nondilated (<30 mm, confirmed with abdominal ultrasound) infrarenal aortas. Neither patients nor controls showed any significant differences in age, sex, or cardiovascular risk factors.

Viborg Study

In 1994, half (4404) of all 65 to 73 year-old males in Viborg County, Denmark, were invited to B-mode-ultrasonographic screening for AAA at their regional hospital. The trial was approved by the respective local scientific ethics committees and reported to the Danish Central Control of Registers.

An AAA was defined as an infrarenal aortic diameter of 30 mm or more, and AAAs >50 mm were referred for surgery. AAAs of 30 to 49 mm were offered yearly follow-up examinations to check for any expansion.[15] Two observers were used, and the arithmetic interobserver variation (2 SD) was 1.4 mm.[16]

The serum samples were left for coagulation at room temperature for 45 minutes before centrifugation. A random sample of 80 cases was used in this study of peroxiredoxin. Follow-up was truncated after 10 years. Sixteen men were referred to planned AAA repair because of expansion (Supplemental Table II). The expansion rate was calculated as the change in the anteriorposterior diameter during the whole observation period, transformed to annual units. The trial was approved by the respective local scientific ethics committees and reported to the Danish Central Control of Registers.

Statistics

ELISA and Western blot results are expressed as mean±SEM. The Wilcoxon paired test was used to analyze differences in PRX-1 levels between luminal and abluminal supernatants of the same samples. The analysis of small and large AAA and control groups was performed with nonparametric tests (Mann-Whitney *U* tests). Pearson correlation analysis was used to examine the univariate correlation between PRX-1 and AAA expansion rate, and it was adjusted for current smoking, body mass index, previous cardiovascular events, use of glucocorticoids, low-dose aspirin, β-blockers, angiotensin coverting enzyme inhibitors, and ankle brachial blood pressure index. The receiving-operating characteristic (ROC) curve analysis was performed to test the predictive clinical value of PRX-1 and initial AAA size, univariately and combined in a multivariate linear regression model concerning prediction of cases expanding more than 1.68 mm/year (mean annual growth). For the analysis of the ROC curve, the null hypothesis was that the test had a performance similar to the diagonal line, ie, the area under the curve was 0.5. If the lowest 95% confidence limit for the area under the curve was more than 0.5, a significant predictive test was said to be present.

Results

DIGE Analysis of ILT Supernatants

Because ILT is involved in AAA evolution and ILT material can be sampled during surgery, it represents a highly relevant model for the study of biological events participating in human AAA. We hypothesized that the proteins differentially released by the biologically active luminal part of the ILT versus the abluminal layer may reflect ILT activity. Proteins released from these different layers (luminal/abluminal) of the ILT of AAA were analyzed by a noncandidate based proteomic strategy using 2D-DIGE (Figure 1). DeCyder analysis revealed 42 differentially expressed spots, and after silver staining of the gels, only visible spots were excised, digested with trypsin, and subjected to MS-based protein identification. Thirty-two proteins out of the 42 spots released differently by the luminal layer compared with the abluminal layer were finally identified by MS (Table and Supplemental Figure I); these proteins are involved in different AAA pathological mechanisms, such as redox balance (eg, peroxiredoxin), inflammatory processes (eg, complement compo-

Table. Proteins Differentially Released From Different Layer Regions (Luminal/Abluminal) of the ILT of Human AAA as Revealed by 2D-DIGE and Identified by MALDI-MS

DeCyder*					Mascot‡			Theoretical Molecular			
Spot	Average Ratio	*t* Test	Protein Description†	Accession Code†	Total Score	Expected	Ion Score	Mass (kDa)/pI	Matched Peptides§	Unmatched Peptides§	Coverage (%)§
1	−2.57	3.81×10^{-2}	Complement component C3	gi 78101268	194	3.10×10^{-13}	NA	114.2/5.55	16	4	18
2	7.35	4.53×10^{-2}	Complement component C3	gi 78101268	230	7.80×10^{-17}	NA	114.2/5.55	20	3	23
3	−2.03	6.97×10^{-3}	Complement component C4A	gi 179674	133	3.90×10^{-7}	NA	189.1/6.7	11	1	7
4	−2.05	4.31×10^{-2}	Complement component C4A	gi 179674	444	5.30×10^{-40}	263	194.3/6.65	17	4	11
5	1.74	4.68×10^{-2}	Transketolase	gi 388891	300	7.70×10^{-24}	83	685.3/7.89	13	1	28
6	−1.96	2.82×10^{-4}	Complement component C3	gi 78101267	642	4.80×10^{-58}	341	71.3/6.82	18	2	43
7	−1.85	2.5×10^{-2}	Transketolase	gi 58176651	209	9.70×10^{-15}	98	56.8/7.16	8	3	22
8	−1.84	2.48×10^{-2}	Phosphoglucomutase 1	gi 21361621	279	9.70×10^{-22}	71	61.7/6.3	13	3	29
9	−1.67	1.85×10^{-2}	Antithrombin III	gi 999513	474	3.10×10^{-41}	321	49.4/5.95	13	3	39
			IGHA1 protein	gi 16741036	272	4.80×10^{-21}	109	54.3/8.07	10	3	26
10	−1.65	8.42×10^{-3}	Prolyl 4-hydroxylase	gi 20070125	113	6.60×10^{-7}	77	57.5/4.76	2	0	6
11	−1.58	4.62×10^{-2}	Dihydropyrimidinase-like 2	gi 4503377	208	1.20×10^{-14}	69	62.7/5.95	9	3	20
12	−1.52	3.67×10^{-2}	Tubulin-β-2c	gi 14124960	148	2.10×10^{-10}	53	26/4.95	5	1	17
13	−1.97	2.38×10^{-2}	Hemopexin	gi 386789	152	8.30×10^{-11}	66	52.2/6.57	5	1	16
14	−1.81	1.27×10^{-2}	Enolase 1	gi 203282367	403	3.90×10^{-34}	213	47.4/6.99	13	4	41
15	−2.14	4.93×10^{-3}	Trypsin-like serine protease	gi 47124562	148	2.10×10^{-10}	77	31.7/8.48	4	1	14
16	1.55	6.04×10^{-4}	γ3-Immunoglobulin	gi 1628395	99	2.70×10^{-5}	52	38.7/8.37	3	2	9
17	1.65	4.15×10^{-2}	Fibrinogen-γ	gi 223170	88	3.20×10^{-4}	NA	33.6/5.98	5	2	22
18	3.88	3.03×10^{-2}	Fibrinogen fragment D	gi 2781208	545	4.20×10^{-50}	165	38.1/5.84	22	3	62
19	4.59	3.97×10^{-2}	Macrophage capping protein	gi 21730367	136	3.30×10^{-9}	85	38.8/5.32	3	2	12
20	−2.08	3.38×10^{-2}	Igγ1	gi 226787	128	1.20×10^{-6}	47	25.6/7	4	1	21
21	−2.14	8.32×10^{-3}	Igγ1	gi 226787	148	1.20×10^{-8}	47	25.6/7	5	1	26
22	−1.74	2.69×10^{-2}	Igγ1	gi 226787	129	1.70×10^{-8}	48	25.6/7	4	1	21
23	−2.13	1.64×10^{-2}	Igγ1	gi 5031410	123	6.60×10^{-8}	59	25.4/6.95	3	0	15
24	−2.87	2.1×10^{-2}	Glyceraldehyde 3-phosphate dehydrogenase	gi 7669492	199	1.70×10^{-15}	98	36.2/8.57	5	0	22
25	−2.67	4.94×10^{-2}	Complement component C4A	gi 443671	430	1.30×10^{-38}	315	195.2/6.79	17	5	8
26	−1.91	3.62×10^{-2}	Phosphatidylinositol transfer protein	gi 5453908	129	1.70×10^{-8}	59	32/6.11	4	0	13
27	−1.58	3.71×10^{-2}	Thrombospondin	gi 553801	216	3.30×10^{-17}	138	42.2/6.6	5	0	14
28	19.01	8.49×10^{-3}	Fibrinogen α-chain	gi 76779196	279	1.70×10^{-23}	100	29.2/9.51	12	5	34
29	7.72	1.84×10^{-2}	Fibrinogen α-chain	gi 76779196	718	2.10×10^{-67}	399	29.2/9.51	21	6	58
30	1.78	1.22×10^{-2}	Peroxiredoxin 1	gi 55959888	134	3.10×10^{-7}	63	10.7/8.79	3	0	34
31	10.61	3.69×10^{-2}	Transgelin	gi 48255905	184	5.30×10^{-14}	74	22.7/8.87	6	2	34
32	−3.15	3.23×10^{-2}	Haptoglobin-α	gi 296653	237	2.60×10^{-19}	139	42.1/6.25	7	3	20

*Protein spot number according to Figure 1 and average volume ratio (luminal/abluminal) as quantified by DeCyder.

†Protein ID and accession number according to the National Center for Biotechnology Information database.

‡Mascot score, expected value, and ion score. NA indicates not applicable. Supplemental Figure I provides annotated MALDI-MS spectra. Ion score applies to peptide MS/MS fragmentation spectra.

§Number of peptides matching and not matching the protein sequence and protein sequence coverage.

nents), extracellular matrix remodeling (thrombospondin), hemostasis (eg, fibrinogen), or heme-related proteins (eg, hemopexin).

PRX-1 Is Mainly Localized and Released by the Luminal Part of AAA Thrombus

Among the differential proteins identified by 2D-DIGE and MS that were found to be altered in the 4 patients and not previously related to AAA, we focused on PRX-1 because regulation of oxidative stress is of major importance in AAA pathophysiology. We validated the levels of PRX-1 in the conditioned media from the same samples used in the DIGE experiment (n=4) and from an additional group of human AAA samples (n=6). PRX-1 release was increased in the luminal layer of the ILT of AAA compared with the abluminal layer, as assessed by ELISA (32±10 versus 10±2 ng/mL,

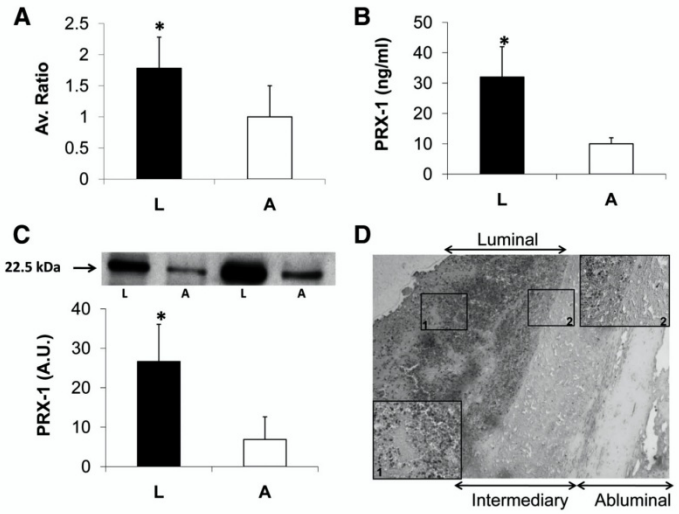


Figure 2. PRX-1 is expressed and released by the luminal part of AAA thrombus. A, Average spot volumes corresponding to luminal (L) (n=4) and abluminal (A) (n=4) samples are shown from the DeCyder software (*average ratio=1.78; $P<0.05$). B, Quantification of PRX-1 levels by ELISA measured in L and A layer supernatants (n=10, *$P<0.001$). C, Representative Western blot from L and A layer supernatants (n=10, *$P<0.001$). D, Immunodetection of PRX-1 in AAA thrombus (magnification, ×10). Inset 1 (magnification, ×20) shows erythrocyte accumulation with leukocyte (nucleated cells) in the luminal part of the thrombus. Inset 2 (magnification, ×20) shows erythrocytes and polynuclear cells positive for PRX-1 at the interface between the luminal an intermediary layers of the thrombus. Positivity is shown in green and nuclei in red. A.U. indicates arbitrary units.

$P<0.05$) and Western blot (Figure 2). Immunodetection of PRX-1 in AAA thrombus (Figure 2D) showed a strong staining in the luminal layer mainly associated with RBCs and polymorphonuclear neutrophils.[17] Both cellular and diffuse staining was observed, which suggests a potential release of PRX-1 in the extracellular compartment. On the contrary, the abluminal layer exhibited a faint staining, in accordance with results obtained by 2D electrophoresis, Western blot, and ELISA.

PRX-1 Is Induced and Released by Oxidative Stress in RBCs

Because the main function of PRX-1 is the inactivation of hydrogen peroxide (H_2O_2), we analyzed H_2O_2 levels in AAA thrombus-conditioned media. Interestingly, H_2O_2 levels were also increased in the luminal layer versus the abluminal layer (17.2 ± 5.4 versus 6.4 ± 1.4 μmol/L, $P<0.05$). In addition, in vitro experiments with isolated RBCs stimulated with H_2O_2 (and lipopolysaccharide) were performed. We have shown that H_2O_2 and lipopolysaccharide induced PRX-1 translocation to the membrane and final release to the conditioned medium (Figure 3). These results suggest that PRX-1 is released as a response to prooxidant molecules present in the thrombus.

Increased PRX-1 Levels in Serum of AAA Patients

This ex vivo approach offers the advantage of identifying proteins potentially released into the bloodstream that could serve as circulating biomarkers for AAA. We measured circulating PRX-1 levels in the serum of patients and controls by ELISA. Results showed that PRX-1 levels were significantly increased in patients with AAA relative to control subjects (14.9 ± 3.5 versus 8 ± 0.6 ng/mL, $P<0.001$, Figure 4A). Because AAA diameter is a surrogate marker of the growth rate, we studied the correlation between circulating PRX-1 levels and AAA diameter. Interestingly, a significant positive correlation between PRX-1 levels and AAA diameter was found ($r=0.6$, $P<0.001$, Figure 4B).

Plasmin-antiplasmin (PAP) complexes and myeloperoxidase (MPO) levels are increased in patients with AAA.[18,19] We confirmed these data in our patient population (data not shown). In addition, we have previously shown that both PAP and MPO levels are increased in the luminal part of the thrombus.[18,19] Interestingly, PRX-1 positively correlated with

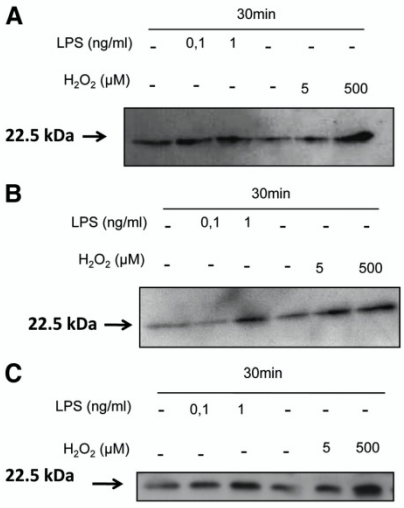


Figure 3. Western blot analysis of PRX-1 in RBCs. Representative Western blots were performed in RBCs isolated from healthy controls and stimulated with lipopolysaccharide (LPS) and H_2O_2 for 30 minutes at different concentrations. A, Total cell lysates. B, Supernatant obtained from cell culture. C, Membrane fractions.

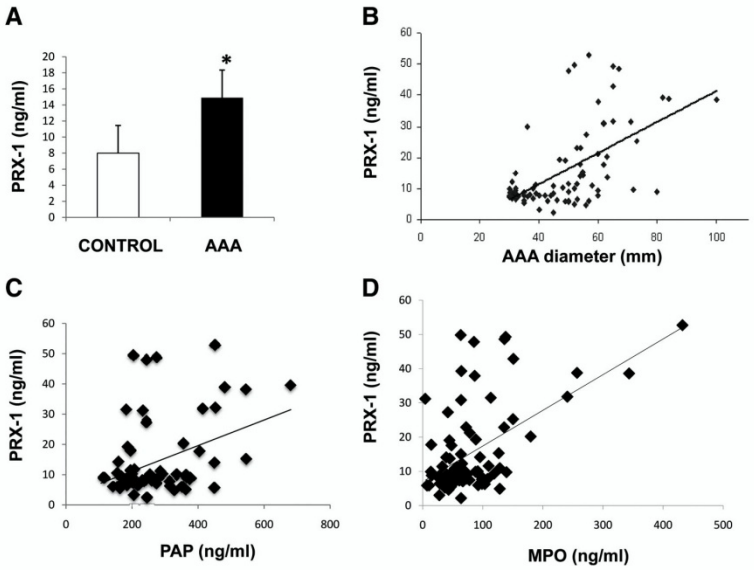


Figure 4. PRX-1 levels in AAA. A, Serum PRX-1 levels were measured from 33 control subjects and 83 patients with AAA (*P<0.001). B, PRX-1 levels correlate with AAA diameter (ρ=0.6; P<0.001, n=83). C and D, A positive correlation was observed between PRX-1 and PAP levels (ρ=0.4, P<0.01, n=83 patients) (D) and between PRX-1 and MPO levels (ρ=0.5, P<0.01, n=83 patients) (D).

both biomarkers of AAA in our patient population (PAP, r=0.4; MPO, r=0.55; P<0.05 for both; Figure 4C and 4D).

PRX-1 Circulating Levels Correlate With AAA Growth

To further extend the results obtained, we measured PRX-1 serum levels in a second cohort of 80 patients from the Viborg study, with a 5-year follow-up, allowing us to test the relevance of PRX-1 as a marker of AAA progression. Similar levels of circulating PRX-1 in small AAA patients (AAA size 3 to 5–5.5 cm) were obtained in both the Viborg and Spanish studies (Viborg=10.4±3.7 ng/mL versus small AAA Spanish=8.3±2.8 ng/mL). Interestingly, the correlation coefficient between PRX-1 levels and AAA growth rate was 0.3 (P=0.01, Figure 5A), persisting after adjustment for current smoking, body mass index, previous cardiovascular events, use of glucocorticoids, low-dose aspirin, β-blockers, angiotensin coverting enzyme inhibitors, and ankle brachial blood pressure index (Supplemental Table III). ROC curve analyses showed that PRX-1 levels were equally valid predictors of

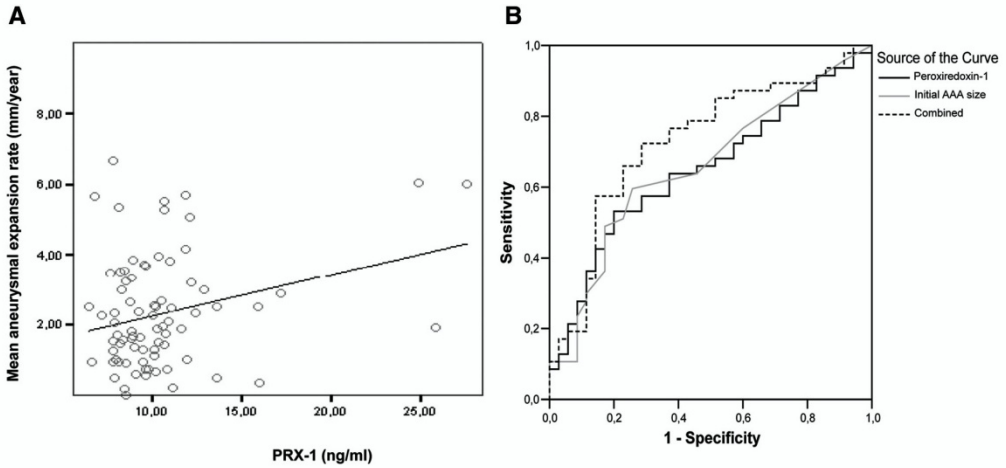


Figure 5. PRX-1 and AAA growth in the Viborg cohort. A, PRX-1 levels were measured from an additional group of serum samples (Viborg cohort). PRX-1 correlates with AAA growth (ρ=0.3; P<0.05, n=80). B, ROC curve for predicting AAA expansion concerning PRX-1 levels and AAA size.

annual expansion rate as initial AAA size exceeding the mean annual growth, with optimal cutoff points with a sensitivity and specificity of 63% and 64%, respectively, for both (Figure 5B). By combining these 2 predictors (PRX-1 and AAA-size) by linear regression to predict annual expansion rate, the optimal validity increased by almost 10% to 72% sensitivity and 71% specificity. Moreover, comparison of the C-statistics showed that the 3 ROC curves differed significantly (χ^2 test=9.11, P=0.01). Consequently, the combination of PRX-1 and size has significantly additive value in predicting growth compared with size alone.

Discussion

Vascular diseases are currently among the most common pathologies, and despite outstanding scientific advances in recent years, the number of effective therapies and useful biomarkers is still very limited, although there is a continuous increase in costs associated with these diseases. Although several potential biomarkers of AAA have been proposed,[2,3] it is essential to discover new biomarkers for early disease detection and risk stratification, which could predict clinical outcome. Moreover, identification of novel biomarkers could help decipher the biological mechanisms leading to progressive dilatation and finally rupture to develop new therapeutic approaches. Our study was performed on the thrombus conditioned medium as a potential source of biomarkers of AAA by a gel-based proteomic approach using DIGE platform. Among the proteins identified by MS, both novel and previously known protein biomarkers for AAA have been unveiled that are associated with some major AAA pathological processes, such as thrombosis and oxidative stress.

The formation of a luminal thrombus may be considered a compensatory mechanism in response to flow perturbations associated with AAA dilatation. The mural thrombus is continuously self-forming at its luminal pole and subjected to proteolysis at the interface with the residual media. Previous studies have shown that thrombus formation and accumulation of leukocytes may have an impact on the structural integrity and stability of the vessel wall and thereby increase the risk of aneurysm rupture.[20] Proteins associated with thrombosis have been the biomarkers most commonly assessed in AAA. In this respect, levels of PAP complexes and D-dimers are elevated in AAA patients and correlate with disease progression.[2,3] Similarly, elevated plasma fibrinogen concentrations predict a greater risk of thrombosis. Interestingly, fibrinogen levels are increased in AAA patients compared with controls, and positive correlations of AAA size, ILT, and fibrinogen concentration are observed.[21] In the present work, among known biomarkers identified, we have shown that fibrinogen/fibrin fragments are abundantly released by the luminal part of the thrombus, in agreement with previous results of our group showing activation of the fibrinolytic system.[19]

Oxidative stress plays a key role in AAA pathophysiology.[22] Although previous studies have mainly addressed the role of oxidative stress in AAA wall, the presence of a mural hemothrombus can also contribute to this process. The trapping of RBCs within ILT may lead to hemolysis and subsequent release of the prooxidant hemoglobin that, when oxidized, transfers heme to endothelium and lipoproteins, thereby enhancing susceptibility to oxidant-mediated injury. Heme potentiates cell cytotoxicity mediated by leukocytes and other sources of reactive oxygen species.[23] Plasmatic hemopexin, haptoglobin, and albumin limit the hemoamplified oxidative damage to the vasculature. In the present work, a decrease in hemopexin and haptoglobin levels was observed in the luminal part of the thrombus, which could favor the prooxidant actions of hemoglobin.

However, oxidative stress is the result of imbalance between prooxidant and antioxidant molecules. In relation to antioxidant enzymes, superoxide dismutase, glutathione peroxidase, and thioredoxin expression levels are increased in AAA tissue.[24,25] Thioredoxin, thioredoxin reductase, and NADPH together constitute a ubiquitous system that regulates cellular redox status. PRX-1 can interact and modulate NADPH activity by inactivating H_2O_2.[26] In this respect, we observed that H_2O_2 levels were increased in the luminal layer versus the abluminal layer, similarly to the tendency observed for PRX-1. In addition, we have shown that H_2O_2 induced PRX-1 translocation to the membrane and final release to the conditioned medium in isolated RBCs. These results suggest that PRX-1 is released as a response to the prooxidant environment present in the ILT.

We hypothesized that the blood compartment could reflect what was observed in the arterial conditioned medium and thus that proteins potentially released into the circulation could serve as biomarkers for the pathology. We show here for the first time that PRX-1 levels are significantly increased in the serum of patients with AAA in relation to controls. Because AAA diameter is a surrogate marker of AAA growth rate and is the clinical parameter used in the management of AAA patients, we studied its potential correlation with PRX-1. We have observed that PRX-1 levels and AAA diameter show a significant positive correlation. Furthermore, a positive correlation among circulating PRX-1, PAP, and MPO levels has been shown, supporting the importance of ILT activities in AAA pathophysiology. Finally, PRX-1 also correlated with AAA growth in a second population with follow-up. Interestingly, by combining both AAA size with PRX-1 levels by linear regression to predict annual expansion rate above the interobserver variation of the measurements,[16] the optimal validity increased almost by 10% up to 72% sensitivity and 71% specificity. The fact that PRX-1 correlates significantly with both AAA size and AAA growth rate in different populations (Spanish and Viborg studies), together with the observed comparable circulating levels in small AAA in the 2 populations, suggests the potential use of PRX-1 as a biomarker for AAA evolution.

On the other hand, despite the fact that PRX-1 levels could be increased in response to the oxidative stress present in AAA, the functional consequences of PRX-1 upregulation are not completely understood. In mammalian cells, high levels of peroxiredoxins are produced, which may account for 0.1% to 0.8% of soluble protein.[27] PRX-1

is overexpressed in response to oxidative stress and has recently been involved in other mechanisms, such as shear stress.[28] Several studies support an antioxidant and anti-apoptotic role for PRX-1 upregulation. PRX-1 was initially described mainly as an antioxidant protein because of its ability to inactivate H_2O_2, $ONOO^-_2$, and other hydroperoxides. However, other cellular roles have been recently proposed for PRX-1, including the modulation of cytokine-induced H_2O_2 levels, which have been shown to mediate the signaling cascade that leads to cell proliferation, differentiation, and apoptosis,[29] and proinflammatory actions.[30] In this respect, PRX-1 has been shown to interact with other proteins and ligands, including hemo and macrophage migration inhibitor factor (MIF).[27,31] MIF is upregulated in AAA[32] and MIF serum levels have been correlated with annual AAA expansion rate and initial AAA size.[33] Interestingly, we have observed a significant strong positive correlation between PRX-1 and MIF in the Viborg sample cohort (not shown). MIF, by modulating the redox status of PRX-1, has been suggested to modulate signaling pathways and glucocorticoid sensitivity.[31] Likewise, we have observed a positive correlation between PRX-1 levels and glucocorticoid treatment.

In the present study, several proteins associated with key mechanisms involved in AAA pathogenesis have been identified by a non-hypothesis-driven proteomic approach. Among them, increased PRX-1 levels have been observed both in conditioned media and in serum from AAA patients. PRX-1 serum levels are associated with AAA size and growth rate, suggesting its possible use as a biomarker for AAA evolution. Additional prospective studies are needed to confirm these results. These results support a main pathological role of oxidative stress in AAA,[22] as well as the potential usefulness of therapies aimed at enhancing antioxidant pathways to prevent AAA progression.[34]

Acknowledgments

We thank Dr Cesar Aparicio for the collection of human samples.

Sources of Funding

This work was supported by the Fighting aneurysmal disease project (FP-7, HEALTH F2-2008-200647 to J-B.M., J.S.L., J.E.), Ministerio de Ciencia e Innovacion (SAF2010/21852 to J.L.M-V., SAF2007/63648 to J.E.), Fundacion Ramon Areces (to L.M.B-C.), CAM (S2006/GEN-0247 to J.E.), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035 to J.E.), PI10/00234 (to L.M.B-C.) and EUS2008-03565 (to J.E.) and Fundacion Pro CNIC (to J.A.L., E Calvo, E Camafeita).

Disclosures

None.

References

1. Sakalihasan N, Limet R, Defawe OD. Abdominal aortic aneurysm. *Lancet*. 2005;365:1577–1589.
2. Urbonavicius S, Urbonaviciene G, Honore B, Henneberg EW, Vorum H, Lindholt JS. Potential circulating biomarkers for abdominal aortic aneurysm expansion and rupture: a systematic review. *Eur J Vasc Endovasc Surg*. 2008;36:273–280.
3. Golledge J, Tsao PS, Dalman RL, Norman PE. Circulating markers of abdominal aortic aneurysm presence and progression. *Circulation*. 2008;118:2382–2392.
4. Blanco-Colio LM, López JA, Martínez-Pinna Albar R, Egido J, Martín-Ventura JL. Vascular proteomics, a translational approach: from traditional to novel proteomic techniques. *Expert Rev Proteomics*. 2009;6:461–464.
5. Nordon I, Brar R, Hinchliffe R, Cockerill G, Loftus I, Thompson M. The role of proteomic research in vascular disease. *J Vasc Surg*. 2009;49:1602–16012.
6. Blanco-Colio LM, Martín-Ventura JL, Muñóz-García B, Orbe J, Páramo JA, Michel JB, Ortiz A, Meilhac O, Egido J. Identification of soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) as a possible biomarker of subclinical atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007;27:916–922.
7. Martin-Ventura JL, Duran MC, Blanco-Colio LM, Meilhac O, Leclercq A, Michel JB, Jensen ON, Hernandez-Merida S, Tuñón J, Vivanco F, Egido J. Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. *Circulation*. 2004;110:2216–2219.
8. Fontaine V, Touat Z, Mtairag el M, Vranckx R, Louedec L, Houard X, Andreassian B, Sebbag U, Palombi T, Jacob MP, Meilhac O, Michel JB. Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus. *Am J Pathol*. 2004;164:2077–2087.
9. Caligiuri G, Rossignol P, Julia P, Groyer E, Mouradian D, Urbain D, Misra N, Ollivier V, Sapoval M, Boutouyrie P, Kaveri SV, Nicoletti A, Lafont A. Reduced immunoregulatory CD31+ T cells in patients with atherosclerotic abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. 2006;26:618–623.
10. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc*. 2006;1:2856–2860.
11. Schuerenberg M, Luebbert C, Eickhoff H, Kalkum M, Lehrach H, Nordhoff E. Prestructured MALDI-MS sample supports. *Anal Chem*. 2000;72:3436–3442.
12. Suckau D, Resemann A, Schuerenberg M, Hufnagel P, Franzen J, Holle A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal Bioanal Chem*. 2003;376:952–965.
13. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 1999;20:3551–3567.
14. Vega de Ceniga M, Esteban M, Quintana JM, Barba A, Estallo L, de la Fuente N, Viviens B, Martin-Ventura JL. Search for serum biomarkers associated with abdominal aortic aneurysm growth: a pilot study. *Eur J Vasc Endovasc Surg*. 2009;37:297–299.
15. Lindholt JS, Juul S, Fasting H, Henneberg EW. Screening for abdominal aortic aneurysms: single centre randomised controlled trial. *BMJ*. 2005;330:750.
16. Lindholt JS, Vammen S, Juul S, Henneberg EW, Fasting H. The validity of ultrasonographic scanning as screening method for abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg*. 1999;17:472–475.
17. Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. *Nature*. 2003;424:561–565.
18. Houard X, Touat Z, Ollivier V, Louedec L, Philippe M, Sebbag U, Meilhac O, Rossignol P, Michel JB. Mediators of neutrophil recruitment in human abdominal aortic aneurysms. *Cardiovasc Res*. 2009;82:532–541.
19. Houard X, Rouzet F, Touat Z, Philippe M, Dominguez M, Fontaine V, Sarda-Mantel L, Meulemans A, Le Guludec D, Meilhac O, Michel JB. Topology of the fibrinolytic system within the mural thrombus of human abdominal aortic aneurysms. *J Pathol*. 2007;212:20–28.
20. Kazi M, Thyberg J, Religa P, Roy J, Eriksson P, Hedin U, Swedenborg J. Influence of intraluminal thrombus on structural and cellular composition of abdominal aortic aneurysm wall. *J Vasc Surg*. 2003;38:1283–1292.
21. Al-Barjas HS, Ariëns R, Grant P, Scott JA. Raised plasma fibrinogen concentration in patients with abdominal aortic aneurysm. *Angiology*. 2006;57:607–614.
22. McCormick ML, Gavrila D, Weintraub NL. Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2007;27:461–469.
23. Balla J, Vercellotti GM, Jeney V, Yachie A, Varga Z, Jacob HS, Eaton JW, Balla G. Heme, heme oxygenase, and ferritin: how the vascular endothelium survives (and dies) in an iron-rich environment. *Antioxid Redox Signal*. 2007;9:2119–2137.

24. Dubick MA, Keen CL, DiSilvestro RA, Eskelson CD, Ireton J, Hunter GC. Antioxidant enzyme activity in human abdominal aortic aneurysmal and occlusive disease. *Proc Soc Exp Biol Med*. 1999;220:39–45.
25. Martinez-Pinna R, Lindholt JS, Blanco-Colio LM, Dejouvencel T, Madrigal-Matute J, Ramos-Mozo P, Vega de Ceniga M, Michel JB, Egido J, Meilhac O, Martin-Ventura JL. Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAA): a potential link of oxidative stress with AAA evolution. *Atherosclerosis*. 2010;212:333–338.
26. Leavey PJ, Gonzalez-Aller C, Thurman G, Kleinberg M, Rinckel L, Ambruso DW, Freeman S, Kuypers FA, Ambruso DR. A 29-kDa protein associated with p67phox expresses both peroxiredoxin and phospholipase A2 activity and enhances superoxide anion production by a cell-free system of NADPH oxidase activity. *J Biol Chem*. 2002;277:45181–45187.
27. Wood ZA, Schroder E, Harris JR, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci*. 2003;28:32–40.
28. Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. *J Biol Chem*. 2008;83:1622–1627.
29. Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science*. 2003;300:650–653.
30. Riddell JR, Wang XY, Minderman H, Gollnick SO. Peroxiredoxin 1 Stimulates Secretion of Proinflammatory Cytokines by Binding to TLR4. *J Immunol*. 2010;184:1022–1030.
31. Kudrin A, Ray D. Cunning factor: macrophage migration inhibitory factor as a redox-regulated target. *Immunol Cell Biol*. 2008;86:232–238.
32. Verschuren L, Lindeman JH, van Bockel JH, Abdul-Hussien H, Kooistra T, Kleemann R. Up-regulation and coexpression of MIF and matrix metalloproteinases in human abdominal aortic aneurysms. *Antioxid Redox Signal*. 2005;7:1195–1202.
33. Pan JH, Lindholt JS, Sukhova GK, Baugh JA, Henneberg EW, Bucala R, Donnelly SC, Libby P, Metz C, Shi GP. Macrophage migration inhibitory factor is associated with aneurysmal expansion. *J Vasc Surg*. 2003;37:628–635.
34. Grigoryants V, Hannawa KK, Pearce CG, Sinha I, Roelofs KJ, Ailawadi G, Deatrick KB, Woodrum DT, Cho BS, Henke PK, Stanley JC, Eagleton MJ, Upchurch GR. Tamoxifen up-regulates catalase production, inhibits vessel wall neutrophil infiltration, and attenuates development of experimental abdominal aortic aneurysms. *J Vasc Surg*. 2005;41:108–114.

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY METHODS

In-gel trypsin digestion

Protein spots from silver-stained gels were visually matched against DIGE images, manually excised from gels, and transferred to pierced V-bottom 96-well polypropylene microplates (Bruker Daltonik, Bremen, Germany) loaded with ultrapure water. Samples were digested automatically using a Proteineer DP protein digestion station (Bruker Daltonik) according to the protocol of Schevchenko et al. (1) with minor variations: gel plugs were submitted to reduction with 10 mmol/l DTT (GE Healthcare, Uppsala, Sweden) in 50 mmol/lammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylation with 55 mmol/l iodoacetamide (Sigma Chemical) in 50 mmol/l ammonium bicarbonate. Gel pieces were then rinsed with 50 mmol/l ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a nitrogen stream. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 7.5 ng/μl in 50 mmol/l ammonium bicarbonate was added to the dry gel pieces, and digestion proceeded at 37ºC for 10 h. The resulting digestion solutions were transferred by centrifugation to V-bottom 96-well polypropylene microplates (Greiner Bio-One, Frickenhausen, Germany), vacuum-dried and kept at 4ºC for later MS analysis.

MALDI Mass spectrometry

Dried samples were redissolved in 0.2 g/l α-cyano-4-hydroxycinnamic acid (Bruker Daltonik) in 50% aqueous acetonitrile and 0.2% trifluoroacetic acid (99.5% purity; Sigma Chemical). This solution was deposited onto a 600 μm AnchorChip prestructured MALDI probe (Bruker Daltonik) (2) and allowed to dry at room temperature. Samples were

automatically analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) (3) with an automated analysis loop controlled by the flexControl 2.2 software (Bruker Daltonik). In a first step, MALDI-MS spectra were acquired by averaging 400 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in a mass range from 800 to 3500 Da. Internal calibration of MALDI-MS mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105. In a second step, precursor ions showing in the MALDI-MS mass spectrum were subject to fragment ion analysis in the tandem (MS/MS) mode to average 1200 spectra. For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. Automated analysis of mass data was performed using the flexAnalysis 2.2 software (Bruker Daltonik). No smoothing or any further spectral processing was applied. MALDI-MS and MS/MS spectra were manually inspected in detail and reacquired, recalibrated and/or relabeled using the aforementioned programs and homemade software when necessary.

MALDI-MS Database searching

MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search a nonredundant protein database (NCBInr 20091015, ~ 10^7 entries, National Center for Biotechnology Information, Bethesda US), using the Mascot software v2.2 (Matrix Science, London, UK; http://www.matrixscience.com) (4). Other relevant search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); allow up to 1 missed cleavage; peptide tolerance ±20 ppm; MS/MS tolerance ±0.5 Da. Protein scores greater than 82 were considered significant ($p<0.05$).

References:

1. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc*. 2006; 1: 2856-2860.

2. Schuerenberg M, Luebbert C, Eickhoff H, Kalkum M, Lehrach H, Nordhoff E. Prestructured MALDI-MS sample supports. *Anal Chem*. 2000; 72: 3436-3442.

3. Suckau D, Resemann A, Schuerenberg M, Hufnagel P, Franzen J, Holle A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal Bioanal Chem* 2003; 376: 952-965

4. Perkins DN, Pappin DJ, Creasy DM; Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* 1999; 20: 3551-3567.

TABLE I (online). *Characteristics of the patients included in the Spanish Cohort Study*

	N	%
Previous hypertension	50	55
Previous dyslipemic	56	65
Previous diabetes	11	14
Previous cardiovascular diseases	32	36
Past/present smokers	41	46
Use of aspirin	33	37
Use of statin	50	55
Use of Ace inhibitors	37	42

TABLE II (online). *Characteristics and events of the included men from The Viborg Cohort Study*

	N	%	
Previous hypertension*	10	12.8	
Previous cerebral ischaemic attack*	4	5.1	
Previous angina pectoris*	12	15.4	
Previous acute myocardial infaction*	20	25.6	
Previous lower limb ischaemia*	5	6.4	
Current smokers	45	57.7	
Use of low dose aspirin	37	47.4	
Use of statin	0	0.0	
Use of Betablockers	15	19.2	
Use of Ace inhibitors	10	12.8	
Planned repair due to progression	16	20.5	
Dead	37	47.4	
	N	**Mean**	**SD**
Age at inclusion	78	68.1	2.9
Systolic brachial blood pressure (mmHg)	78	157.8	21.6
Diastolic brachial blood pressure (mmHg)	78	92.6	13.2
Ankle brachial blood pressure index	78	0.99	0.24
Body Mass Index (kg/m^2)	78	27.0	3.9
Initial AAA-size (mm)	78	33.4	4.0
Annual aneurismal growth rate (mm/year)	78	2.22	1.56

* Identified by national-wide registry of hospital-diagnoses at discharge from hospital

Table III (online). *Multivariant analysis performed on Viborg cohort study*

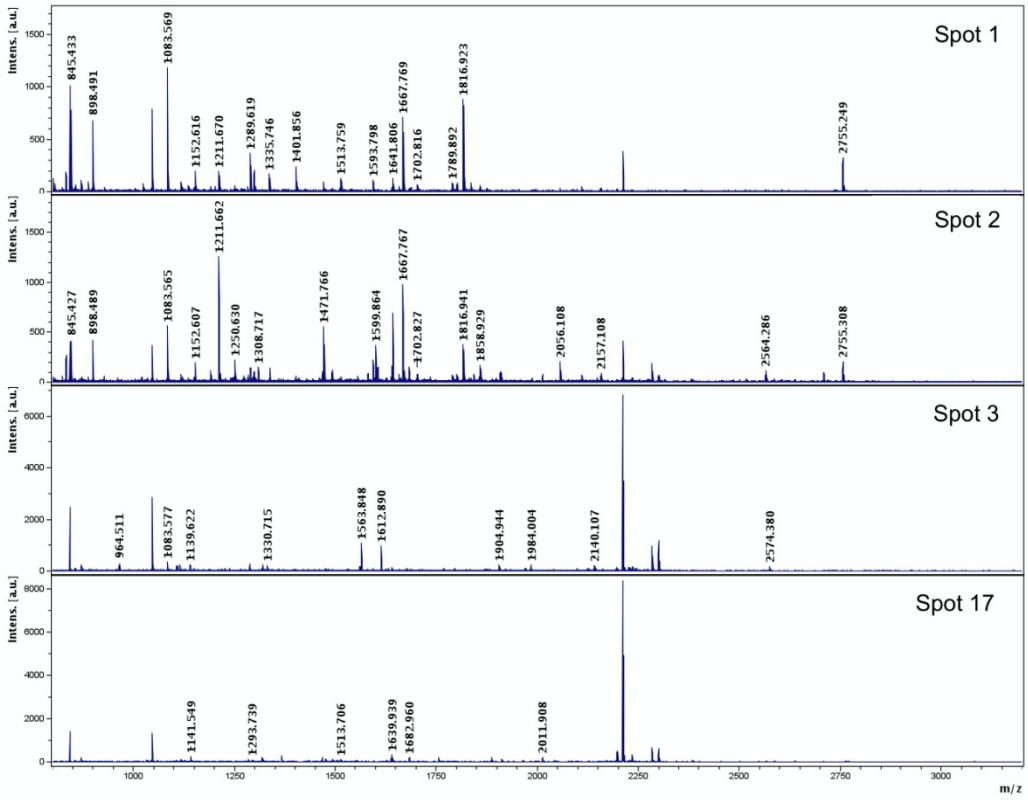
Coefficients(*a*)

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	-4,997	2,096		-2,384	0,02
Peroxiredoxin-1	0,106	0,048	0,251	2,21	0,031
Initial AAA-size	0,142	0,045	0,354	3,147	0,002
Current smoking	0,936	0,378	0,287	2,48	0,016
Use of glucocorticoids	-0,344	0,58	-0,069	-0,593	0,555
Body mass index	0,001	0,047	0,001	0,013	0,99
Coexisting cardiovascular disease	0,348	0,409	0,107	0,85	0,398
Ankle brachial bloodpressure index	0,783	0,748	0,117	1,047	0,299
Use of β-blocker	-0,167	0,516	-0,041	-0,323	0,747
Use of ACE-inhibitor	0,468	0,56	0,098	0,836	0,406
Use of low-dose aspirin	-0,048	0,374	-0,015	-0,128	0,899

a Dependent Variable: Mean annual aneurismal expansion rate (mm/year)

SUPPLEMENTARY FIGURE.

Supplementary Figure I. MALDI-MS spectra corresponding to proteins identified without MS^2 data.



Supplementary Figure 1. MALDI-MS spectra corresponding to proteins identified without MS^2 data.

Table III (online). *Multivariant analysis performed on Viborg cohort study*

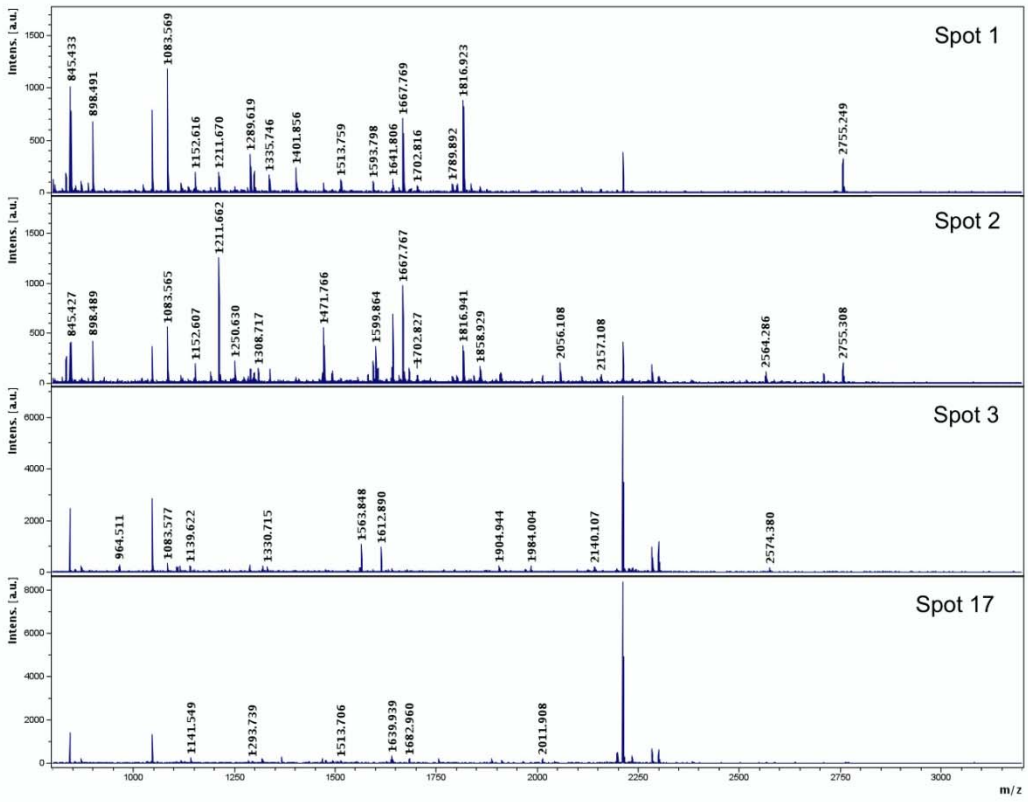
Coefficients(*a*)

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	-4,997	2,096		-2,384	0,02
Peroxiredoxin-1	0,106	0,048	0,251	2,21	0,031
Initial AAA-size	0,142	0,045	0,354	3,147	0,002
Current smoking	0,936	0,378	0,287	2,48	0,016
Use of glucocorticoids	-0,344	0,58	-0,069	-0,593	0,555
Body mass index	0,001	0,047	0,001	0,013	0,99
Coexisting cardiovascular disease	0,348	0,409	0,107	0,85	0,398
Ankle brachial bloodpressure index	0,783	0,748	0,117	1,047	0,299
Use of β-blocker	-0,167	0,516	-0,041	-0,323	0,747
Use of ACE-inhibitor	0,468	0,56	0,098	0,836	0,406
Use of low-dose aspirin	-0,048	0,374	-0,015	-0,128	0,899

a Dependent Variable: Mean annual aneurismal expansion rate (mm/year)

SUPPLEMENTARY FIGURE.

Supplementary Figure I. MALDI-MS spectra corresponding to proteins identified without MS^2 data.



Supplementary Figure 1. MALDI-MS spectra corresponding to proteins identified without MS^2 data.

Cardiovascular Research (2011) **89**, 225–233
doi:10.1093/cvr/cvq278

TWEAK–*Fn14* interaction enhances plasminogen activator inhibitor 1 and tissue factor expression in atherosclerotic plaques and in cultured vascular smooth muscle cells

Begoña Muñoz-García[1], Julio Madrigal-Matute[1], Juan A. Moreno[1], Jose L. Martin-Ventura[1], Oscar López-Franco[1], Cristina Sastre[1], Luis Ortega[2], Linda C. Burkly[3], Jesús Egido[1], and Luis M. Blanco-Colio[1]*

[1]Renal and Vascular Research Laboratory, Instituto de Investigación Sanitaria, Fundación Jiménez Díaz (IIS-FJD), Autonoma University, Avd Reyes Católicos 2, 28040 Madrid, Spain; [2]Hospital Clínico, Madrid, Spain; and [3]Biogen Idec, Cambridge, MA, USA

Received 16 March 2010; revised 4 August 2010; accepted 23 August 2010; online publish-ahead-of-print 1 September 2010

Time for primary review: 14 days

Aims Atherosclerotic plaque development can conclude with a thrombotic acute event triggered by plaque rupture/erosion. Tumour necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the tumour necrosis factor superfamily that, through its receptor, fibroblast growth factor-inducible 14 (*Fn14*), participates in vascular remodelling, increasing vascular inflammatory responses and atherosclerotic lesion size in *ApoE* knockout mice. However, the role of the TWEAK–*Fn14* axis in thrombosis has not been previously investigated.

Methods and results We have examined whether TWEAK regulates expression of prothrombotic factors such as tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) in atherosclerotic plaques as well as in human aortic vascular smooth muscle cells (hASMCs) in culture. Expression of TF and PAI-1 was colocalized and positively correlated with *Fn14* in human carotid atherosclerotic plaques. *In vitro*, TWEAK increased TF and PAI-1 mRNA, protein expression and activity in hASMCs. All these effects were reversed using blocking anti-TWEAK monoclonal antibody, anti-*Fn14* antibody or *Fn14* small interfering RNA, indicating that TWEAK increased the prothrombotic state through its receptor, *Fn14*. Finally, *ApoE*$^{-/-}$ mice were fed a hyperlipidaemic diet for 10 weeks, then randomized and treated with saline (controls), TWEAK (10 μg/kg/day), anti-TWEAK neutralizing monoclonal antibody (1000 μg/kg/day), or non-specific immunoglobulin G (1000 μg/kg/day) daily for 9 days. Systemic TWEAK injection increased TF and PAI-1 protein expression in the aortic root of *ApoE*$^{-/-}$ mice. Conversely, TWEAK blocking antibodies diminished both TF and PAI-1 protein expression compared with non-specific immunoglobulin G-treated mice.

Conclusions Our results indicate that the TWEAK–*Fn14* axis can regulate activation of TF and PAI-1 expression in vascular cells. TWEAK–*Fn14* may be a therapeutic target in the prothrombotic complications associated with atherosclerosis.

Keywords TWEAK • Atherosclerosis • Plasminogen activator inhibitor 1 • Tissue factor • Coagulation

1. Introduction

Tumour necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the tumour necrosis factor (TNF) family that binds to its receptor, fibroblast growth factor-inducible 14 (*Fn14*), to induce a large number of physiological and pathological processes depending on cell type and environment. TWEAK is expressed in many tissues and cells, while *Fn14* is expressed at relatively low levels in normal conditions and highly regulated by cytokines[1] and growth factors.[2–4] Several *in vivo* studies have revealed an increased expression of both TWEAK and Fn14 after tissue injury,[3–7] suggesting a role of the TWEAK–*Fn14* axis in both tissue injury and repair. In

* Corresponding author. Tel: +34915504800, Fax: +34915442636, Email: lblanco@fjd.es

relation to vascular diseases, TWEAK and *Fn14* are expressed in human atherosclerotic plaques.[1] TWEAK enhances vascular lesions associated with hyperlipidaemia in apolipoprotein E (*ApoE*) knockout mice by exacerbating secretion of pro-inflammatory cytokines and macrophage cell recruitment.[8] TWEAK and *Fn14* are also implicated in several processes in vascular cells, such as proliferation and migration,[2,9–10] and expression of adhesion molecules,[9] metalloproteinases,[11–12] or cytokines,[1] monocyte cytotoxicity,[13] apoptosis,[14] and angiogenesis.[10] However, there is no information about the possible implication of the TWEAK–*Fn14* axis for thrombosis. Plaque rupture or erosion, and subsequent thrombosis, represent the main complications of atherosclerosis that could lead to an acute cardiovascular event. Different molecules, such as plasminogen activator inhibitor1 (PAI-1) and tissue factor (TF), are responsible of haemostasis and thrombosis.[15–16] Tissue factor is the principal initiator of the clotting cascade, while PAI-1 plays a critical role in inhibiting fibrinolysis, and thereby the activity of both molecules promotes thrombotic states and plays a crucial role in vascular diseases.[17–18]

Tissue factor is a 45–50 kDa transmembrane glycoprotein that binds to factor VII and VIIa and forms a complex that induces the conversion of factor IX and X to IXa and Xa, respectively, leading to thrombin formation and insoluble fibrin deposition. Although there is a variety of cells that express TF, macrophages and smooth muscle cells (SMCs) are the major source of TF within atherosclerotic plaque.[19] Tissue factor is present in three cellular pools in SMCs, namely surface TF (active), encrypted TF, and intracellular TF, and it can be released from SMCs in microparticles.[20] Basal expression of TF is low in SMCs, but it could be increased by different molecules, such as the cytokines monocyte chemotactic protein-1 (MCP-1), CD40 ligand, and TNF-α or platelet-derived growth factor, angiotensin II, nitric oxide, and aggregated low-density lipoproteins.[18] In addition, the amount of TF within atherosclerotic plaque modulates its thrombogenicity.[21]

In contrast, PAI-1 is a member of the serpin superfamily proteinase inhibitors that inhibits plasminogen activator, urokinase-type plasminogen activator, and α-thrombin, decreasing dissolution of fibrin clots by the fibrolytic system. Plasminogen activator inhibitor 1 is present in healthy arteries, where is expressed by endothelial cells and SMCs. In atherosclerotic plaques, PAI-1 can be detected by electron microscopy in SMCs, macrophages, and extracellular areas. Expression of PAI-1 in SMCs within the fibrous cap is increased compared with SMCs located in the adjacent media or in normal arterial tissue, and its expression is increased during vascular damage.[22]

In the present study, we examined the effect of TWEAK–*Fn14* binding on TF and PAI-1 expression and activity in cultured human aorta vascular SMCs (hASMCs). In addition, we analysed the association of both proteins with *Fn14* in human carotid atherosclerotic plaques. Furthermore, we have analysed the effect of recombinant TWEAK or anti-TWEAK monoclonal antibody (mAb) treatment on atherosclerotic plaques of hyperlipidaemic *ApoE* knockout mice.

2. Methods

2.1 Reagents

Ham's F-12, penicillin, streptomycin, and trypsin–EDTA were obtained from BioWhittaker (Verviers, Belgium). Fetal bovine serum was from Gibco (Paisley, UK). Recombinant human TWEAK was from Alexis (Plymouth Meeting, PA, USA). A blocking murine anti-TWEAK mouse mAb (clone P2D10) was generated in *TWEAK*$^{-/-}$ mice and cross-reacts with human TWEAK. The generation of mAb and TWEAK blocking activity was previously described.[7] This antibody and non-specific IgG were generously gifted by Biogen Idec. (Cambridge, MA, USA). Mouse anti-human *Fn14* (ITEM-2) was from eBioscience (San Diego, CA, USA). The *Fn14* and control small interfering RNA (siRNA) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Invitrogen (Paisley, UK), respectively. The remaining reagents were obtained from Sigma (St. Louis, MO, USA) unless specified otherwise.

2.2 Cell culture

Human aortic smooth muscle cells [hASMCs; American Type Culture Collection (Virginia, USA); CRL-1999] were cultured in Ham's F-12 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, ITS (2.5 μg/mL insulin from bovine pancreas, 2.5 μg/L human transferrin, 2.5 ng/mL sodium selenite), and 30 μg/mL endothelial growth supplement, at 37°C in air supplemented with 5% CO_2. Cells between passages 3 and 7 were used for all experiments. These cells were shown to express *Fn14*, consistent with our previous publication.[1]

2.3 Extraction of RNA and real-time PCR

Total RNA from hASMCs was obtained by TRIzol method (Life Technologies, Carlsbad, CA, USA) and quantified by absorbance at 260 nm in duplicate. Real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated *Taq*DNA polymerase (Amplitaq Gold). After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. GADPH rRNA served as housekeeping gene and was amplified in parallel with the genes of interest. The expression of target genes was normalized to housekeeping transcripts. Target gene, forward and reverse primers, and probes were designed using Primer Express 1.5 software (Applied Biosystems, Carlsbad, CA, USA). All primers, probes, and reagents were obtained from Applied Biosystems (Carlsbad, CA, USA). All measurements were performed in duplicate. Values of each sample were obtained as multiples of their baseline values.

2.4 Western blot

Cells from different experimental conditions were collected and pelleted. Western blots of cellular or supernatant proteins were analysed as previously described.[23] The blots were incubated with anti-human tissue factor antibody (American Diagnostica Inc., Stamford, CT, USA) or anti-human PAI-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rehybridated with anti-α-tubulin mAb (B-5 to 1–2; Sigma, St. Louis, MO, USA) to confirm equal loading and transfer of proteins. Quantification was expressed as arbitrary densitometric units (ADU).

2.5 Tissue factor and PAI-1 activity assays

Activity of PAI-1 in the supernatants of cell cultures was determined using AssaySense Human PAI-1 Chromogenic Activity Assay Kit (AssayPro, St Charles, MO, USA). For TF activity determination, cells were lysed by sonication in a buffer of 50 mM Tris–HCl, 100 mM NaCl, and 0.1% Triton X-100, pH7.4. Tissue factor procoagulant activity was quantificated in cell lysates by Actichrome TF (American Diagnostica Inc., Stamford, CT, USA), according to the manufacter's instructions.

2.6 Animal model

Twenty-four *ApoE* knockout mice (16 weeks of age) were fed on a hyperlipidaemic diet (21.2% fat (0.15% cholesterol) + 16.7% proteins) for 10 weeks. After that, animals were randomized into four groups: mice injected i.p. with saline (control group = 6), human recombinant TWEAK (10 μg/kg/day, $n = 6$), anti-TWEAK mAb (1000 μg/kg/day, $n = 6$), or non-specific immunoglobulin G (IgG; 1000 μg/kg/day, $n = 6$) daily for 9 days.

The dose of TWEAK was chosen based on prior *in vivo* studies,[7] and the dose of anti-TWEAK mAb was chosen to be 100-fold in excess of the

TWEAK dose. Anaesthetized mice were saline perfused. The aorta was embedded in OCT and frozen for immunohistochemistry. The study was performed in accordance with the European Union normative and was approved by the ethical committee of our institution. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996).

2.7 Patients

Twenty-six patients undergoing carotid end-arterectomy at Hospital Clínico (Madrid, Spain) with asymptomatic carotid stenosis (>70%) were included in the study (21 men and five women, 70 ± 7 years old, 34% hyperlipidaemic, 26% diabetic). The region of the bifurcation of the common carotid artery was chosen. Atherosclerotic plaques were collected at the time of the surgery. Informed consent was obtained before enrolment in all cases. The study was approved by the hospital's ethics committee (Hospital Clínico, Madrid, Spain) according to the institutional and the Good Clinical Practice guidelines. The study was performed in accordance with the principles outlined in the Declaration of Helsinki, and all participants gave written informed consent.

2.8 Immunochemistry

Carotid atherosclerotic plaques were stored in paraformaldehyde for 24 h and later in ethanol until paraffin embedded.

Plaques were cross-sectioned into 5-μm-thick pieces, dewaxed and rehydrated, and incubated overnight with goat anti-human TF (1:100 dilution; American Diagnostica Inc., Stamford, CT, USA), mouse anti-human PAI-1 (1:100 dilution, SC-8979; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit anti-human *Fn14* (1:200 dilution; Cell Signaling Technology, Danvers, MA, USA). For colocalization studies, immunofluorescence was carried out on slides after performing immunohistochemistry for TF, PAI-1, or SMCs. Negative controls using the corresponding IgG

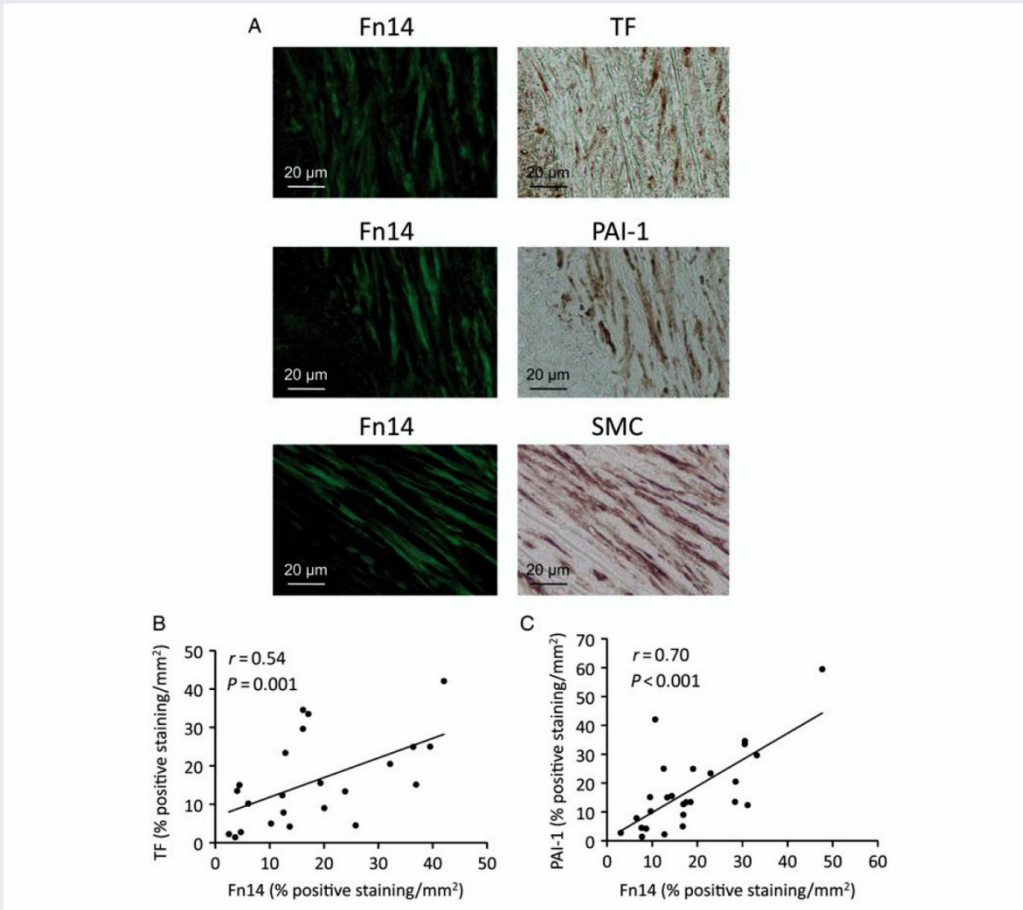


Figure 1 Expression of PAI-1 and TF is associated with *Fn14* in human carotid atherosclerotic plaques. (*A*) Colocalization of *Fn14* (green) and TF, PAI-1 or SMCs (brown). Pearson correlation showing the positive association between TF and *Fn14* (*B*) or PAI-1 and *Fn14* expression (*C*) in 26 human carotid atherosclerotic plaques.

were included to check for non-specific staining. Biotinylated secondary antibodies were applied for 1 h. Then avidin–biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) was added for 30 min. Sections were stained with 3,3'-diaminobenzidine (DAKO, Glostrup, Denmark), counterstained with haematoxylin, and mounted in Pertex.

Aortic roots were cross-sectioned into 4-μm-thick sections. Primary antibodies were rabbit anti-mouse TF (1:200 dilution; American Diagnostica Inc., Stamford, CT, USA) and rabbit anti-PAI-1(1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Donkey anti-rabbit biotin was used as secondary antibody and then ABComplex–horseradish peroxidase was added. The immunohystochemistry sections were stained with 3,3'-diaminobenzidine (DAKO, Glostrup, Denmark), counterstained with haematoxylin, and mounted in Kaisers Glyceringelatine (Merck, Nottingham, UK).

2.9 Quantification

Computer-assisted morphometric analysis with the Olympus semi-automatic image analysis system Micro Image software (version 1.0 for Windows) was performed by a pathologist who was blinded to the group of patients to which the atherosclerotic plaques belonged, as previously described.[1] Results are expressed as the per mm^2 for human plaques or per μm^2 for animal plaques.

2.10 Statistical analysis

Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as means ± SEM. Significance at the $P < 0.05$ level was assessed by Student's *t*-test for two groups of data and ANOVA for three or more groups. *In vitro* experiments were replicated

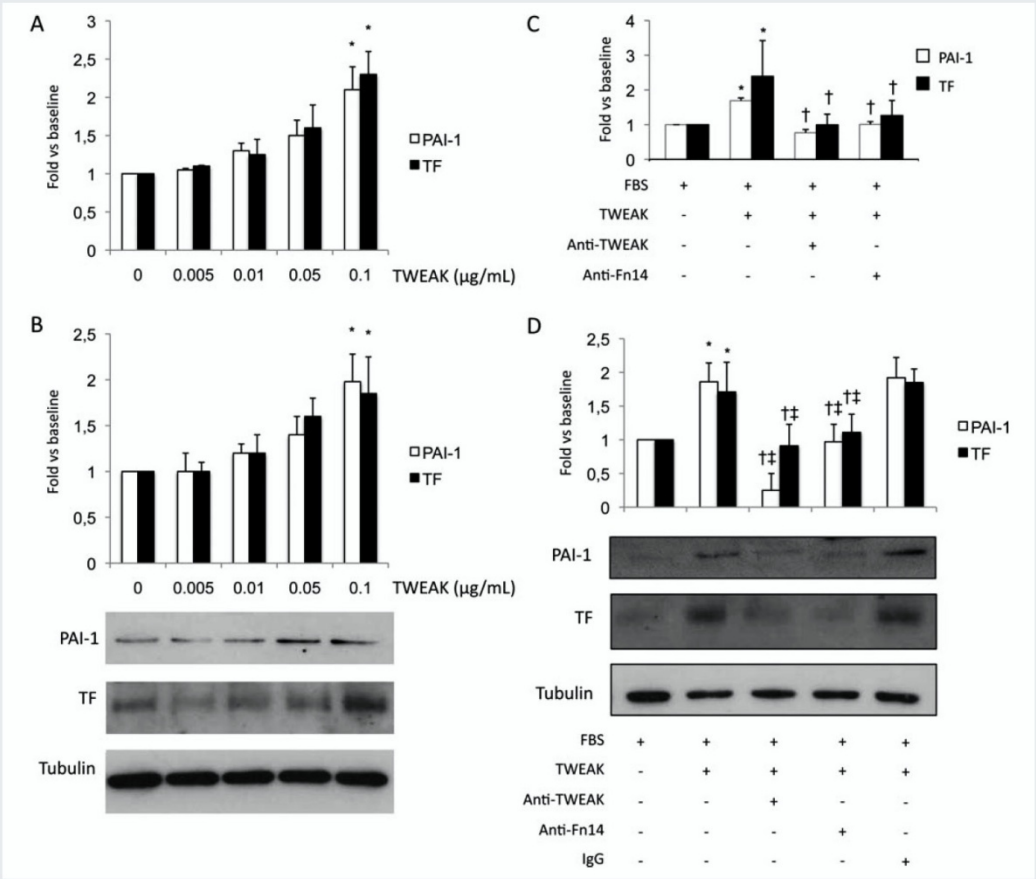


Figure 2 TWEAK increases PAI-1 and TF expression in hASMCs. Quantitative real-time PCR analysis of PAI-1 and TF mRNA (18 h; *A*) and protein expression (24 h; *B*) in hASMCs treated with different concentrations of recombinant TWEAK. Quantitative real-time PCR analysis of PAI-1 and TF mRNA (18 h; *C*) and protein expression (24 h; *D*) in hASMCs treated with 0.1 μg/mL of recombinant TWEAK alone or in combination with anti-TWEAK (10 μg/mL), anti-*Fn14* antibodies (5 μg/mL), or non-specific IgG (10 μg/mL). Values are normalized to GAPDH expression, and results are expressed as multiples of control values. Values shown are means ± SD of three independent experiments. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. TWEAK; and $^{\ddagger}P < 0.05$ vs. IgG.

three times for each incubation period. Differences were considered significant at a value of $P < 0.05$.

3. Results

3.1 Association between *Fn14* expression and PAI-1 or TF content in human carotid atherosclerotic plaques.

We have previously observed that *Fn14* is only expressed in injured vessel wall,[1] reflecting that its expression is related to the pathological tissue remodelling. However, TWEAK expression is observed in both healthy vessels and atherosclerotic plaques at similar levels. This suggests, therefore, that *Fn14* is the regulated member of the TWEAK–*Fn14* axis within vessel wall. Taking these data into account, we have analysed the possible relationship between *Fn14* and PAI-1 or TF expression in atherosclerotic plaques. *Fn14* colocalized with TF, PAI-1, and SMCs within atherosclerotic plaques (*Figure 1A*). In addition, *Fn14*, PAI-1, and TF expression in serial sections of atherosclerotic plaques revealed a highly positive linear correlation between *Fn14* and TF ($r = 0.54$; $P < 0.001$; *Figure 1B*) or *Fn14* and PAI-1 expression ($r = 0.70$; $P < 0.001$; *Figure 1C*). These results could indicate that the TWEAK–*Fn14* axis participates in the regulation of PAI-1 and TF expression in the vascular wall.

3.2 TWEAK induces PAI-1 and TF expression in cultured hASMCs

We have studied whether TWEAK can modulate PAI-1 and TF mRNA and protein expression in cultured hASMCs. For that purpose, hASMCs were cultured in the absence or presence of TWEAK (0.1 μg/mL), and PAI-1 and TF mRNA or proteins were analysed. Real-time PCR revealed that TWEAK increased PAI-1 and TF mRNA expression in a dose- (*Figure 2A*) and time-dependent manner (not shown), peaking at 18 h for PAI-1 and for TF (*Figure 2A*).

Tissue factor is predominantly expressed on the cell surface where it can be active,[17] while newly synthesized PAI-1 is deposited by SMCs first in the extracellular matrix, and is later released from the extracellular matrix to conditioned medium.[24] For these reasons, we have analysed TF expression in cell lysates and PAI-1 secretion in culture supernatants.

TWEAK induced TF protein expression in cultured hASMCs as well as PAI-1 content in culture medium in a dose-dependent manner (*Figure 2B*).

3.3 *Fn14* is implicated in TF and PAI-1 expression induced by TWEAK

To delineate the implication of *Fn14* expression in the signalling mechanism underlying TF and PAI-1 induction by TWEAK, we have analysed the effect of anti-*Fn14* and anti-TWEAK blocking antibodies on PAI-1 and TF expression induced by TWEAK. Pre-incubation with blocking antibodies against TWEAK or *Fn14* prevented PAI-1 and TF mRNA expression induced by TWEAK (*Figure 2C*). In addition, anti-TWEAK or anti-*Fn14* blocking antibodies also diminished PAI-1 release and TF protein expression (*Figure 2D*). No effect was observed when cells were incubated with non-specific IgG (*Figure 2D*).

To further investigate the role of *Fn14* in PAI-1 and TF mRNA induction by TWEAK, we analysed PAI-1 and TF mRNA expression in *Fn14* siRNA-transfected hASMCs. Silencing of *Fn14* completely prevented both PAI-1 and TF mRNA expression induced by TWEAK in hASMCs (*Figure 3A*). Similar results were observed when we studied TF protein expression and PAI-1 release into the culture medium (*Figure 3B*). No effect was observed when cells were incubated with control siRNA (*Figure 3B*).

3.4 Activity of PAI-1 and TF is increased in TWEAK-treated hASMCs

With the purpose to analyse whether the increment in PAI-1 and TF expression induced by TWEAK is reflected in their procoagulation functions, we analysed both TF activity in total cell extract and PAI-1 activity in supernatants of cultured hASMCs. TWEAK-treated cells showed an increase in TF activity (324 ± 23 vs. 245 ± 28 pM,

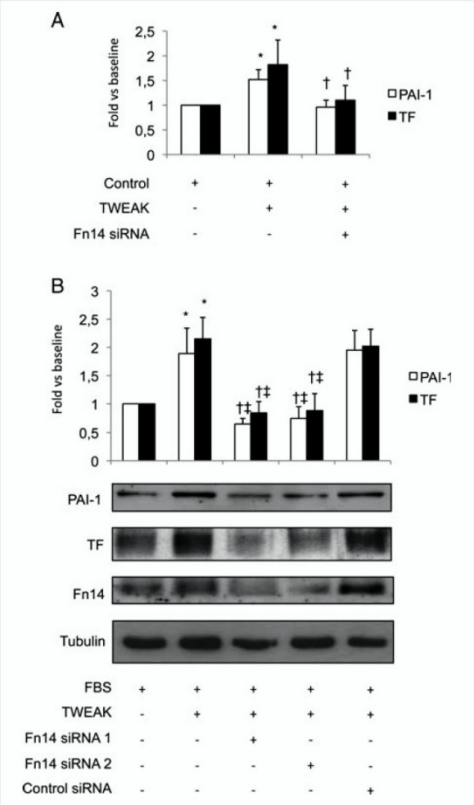


Figure 3 *Fn14* mediates PAI-1 and TF expression induced by TWEAK in hASMCs. Quantitative real-time PCR analysis of PAI-1 and TF mRNA (18 h; *A*) and protein expression (24 h; *B*) in hASMCs treated with 0.1 μg/mL of recombinant TWEAK alone or in combination with two different *Fn14* siRNAs [*Fn14* siRNA 1 (Invitrogen) or *Fn14* siRNA 2 (Santa Cruz Biotec.)]. Values were normalized to GADPH expression, and results are expressed as multiples of control values. Values shown are means ± SD of three independent experiments. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. TWEAK; and $^{\ddagger}P < 0.05$ vs. control siRNA.

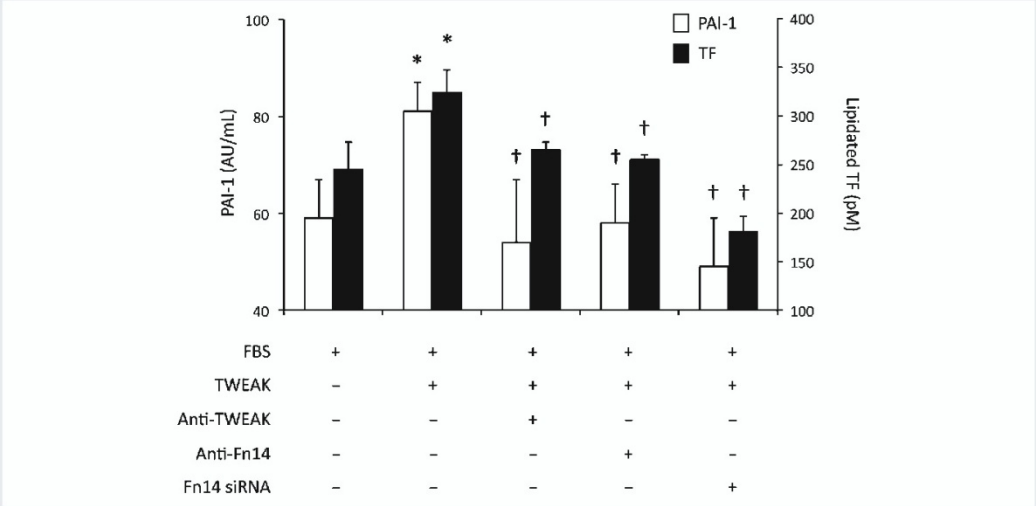


Figure 4 TWEAK increases PAI-1 and TF activity in hASMCs. PAI-1 and TF activity in hASMCs treated with 0.1 μg/mL of recombinant TWEAK alone or in combination with anti-TWEAK (10 μg/mL), anti-*Fn14* antibodies (5 μg/mL), or *Fn14* siRNA after 24 h. Values shown are means ± SD of three independent experiments. *$P < 0.05$ vs. control; and †$P < 0.05$ vs. TWEAK.

$P < 0.05$), which was reversed by anti-TWEAK (265 ± 8 vs. 324 ± 23 pM, $P < 0.05$), and by anti-*Fn14* antibodies (255 ± 5 vs. 324 ± 23 pM, $P < 0.05$), as well as by inhibition of *Fn14* expression with *Fn14* siRNA (180 ± 16 vs. 324 ± 23 pM, $P < 0.05$; *Figure 4*).

Similar results were obtained when PAI-1 activity was analysed. TWEAK increased PAI-1 activity in hASMC culture medium (82 ± 6 vs. 59 ± 8 AU/mL, $P < 0.05$). Blocking TWEAK–*Fn14* signalling by antibodies against TWEAK or *Fn14* resulted in a reduction in PAI-1 activity (54 ± 13 vs. 82 ± 6 AU/mL, $P < 0.05$ and 59 ± 8 vs. 82 ± 6 AU/mL, $P < 0.05$, respectively). Treatment with *Fn14* siRNA also reduced PAI-1 in hASMC culture supernatants (56 ± 2 vs. 82 ± 6 AU/mL, $P < 0.05$ and 49 ± 10 vs. 82 ± 6 AU/mL, $P < 0.05$, respectively; *Figure 4*).

3.5 TWEAK modulates PAI-1 and TF expression in atherosclerotic plaques from *ApoE* knockout mice

We have previously demonstrated that TWEAK injection increases inflammation and lesion size in hyperlipidaemic *ApoE* knockout mice.[6] Using the same animal model, we observed that animals injected with TWEAK showed an increment in PAI-1 expression in atherosclerotic plaques presented in the aortic root compared with animals injected with saline or non-specific IgG [9.3 ± 3.3 (TWEAK) vs. 5.5 ± 2.1 (Control), $P < 0.05$; or vs. 6.1 ± 2.0 (IgG), $P < 0.05$; *Figure 5*]. In addition, mice treated with an anti-TWEAK mAb showed a reduced PAI-1 expression compared with control and non-specific IgG-injected mice [2.7 ± 1.2 (Anti-TWEAK) vs. 5.5 ± 2.1 (Control), $P < 0.05$;or vs. 6.1 ± 2.0 (IgG), $P < 0.05$]. Concordantly, TWEAK injection increased TF expression in the aortic root of *ApoE*$^{-/-}$ mice compared with mice treated with saline or non-specific IgG [9.9 ± 3.2 (TWEAK) vs. 5.3 ± 1.5 (Control), $P < 0.05$; or vs. 6.0 ± 2.3 (IgG), $P < 0.05$; *Figure 5*]. Moreover, TF expression decreased after anti-TWEAK mAb treatment compared with saline- or non-specific IgG-treated animals [2.9 ± 1.4 (anti-TWEAK) vs. 5.3 ± 1.5 (Control), $P < 0.05$; or vs. 6.0 ± 2.3 (IgG), $P < 0.05$]. Furthermore, western blot from the whole aortas showed similar results to those observed in the aortic root (*Figure 5B*). These results suggest that endogenous TWEAK may play a role in PAI-1 and TF expression within the arterial wall *in vivo*.

4. Discussion

In the present work, we have shown that *Fn14* is colocalized with both PAI-1 and TF within human carotid atherosclerotic plaques. Furthermore, TWEAK administration induces PAI-1 and TF expression in cultured hASMCs and in atherosclerotic plaques of hyperlipidaemic *ApoE*$^{-/-}$ mice. The involvement of endogenous TWEAK in plaque thrombogenicity was also demonstrated by reduced PAI-1 and TF expression after short-term treatment with anti-TWEAK neutralizing mAb in *ApoE*$^{-/-}$ mice.

TWEAK has been described as a mediator of many processes within the arterial wall. It is involved in development of atherosclerosis by inducing inflammation, apoptosis, proliferation, and angiogenesis. We and others have observed that *Fn14* expression is highly regulated in vascular tissue, with its expression being very low in healthy vessels[1–4] and increased in injured vessels, indicating that the TWEAK–*Fn14* axis could be implicated in vascular remodelling. Tissue factor and PAI-1 expression are also increased in atherosclerotic plaques in comparison with normal vessels,[21,25] and we have observed a positive association between *Fn14* and PAI-1 or TF in human atherosclerotic plaques. These data could indicate that the

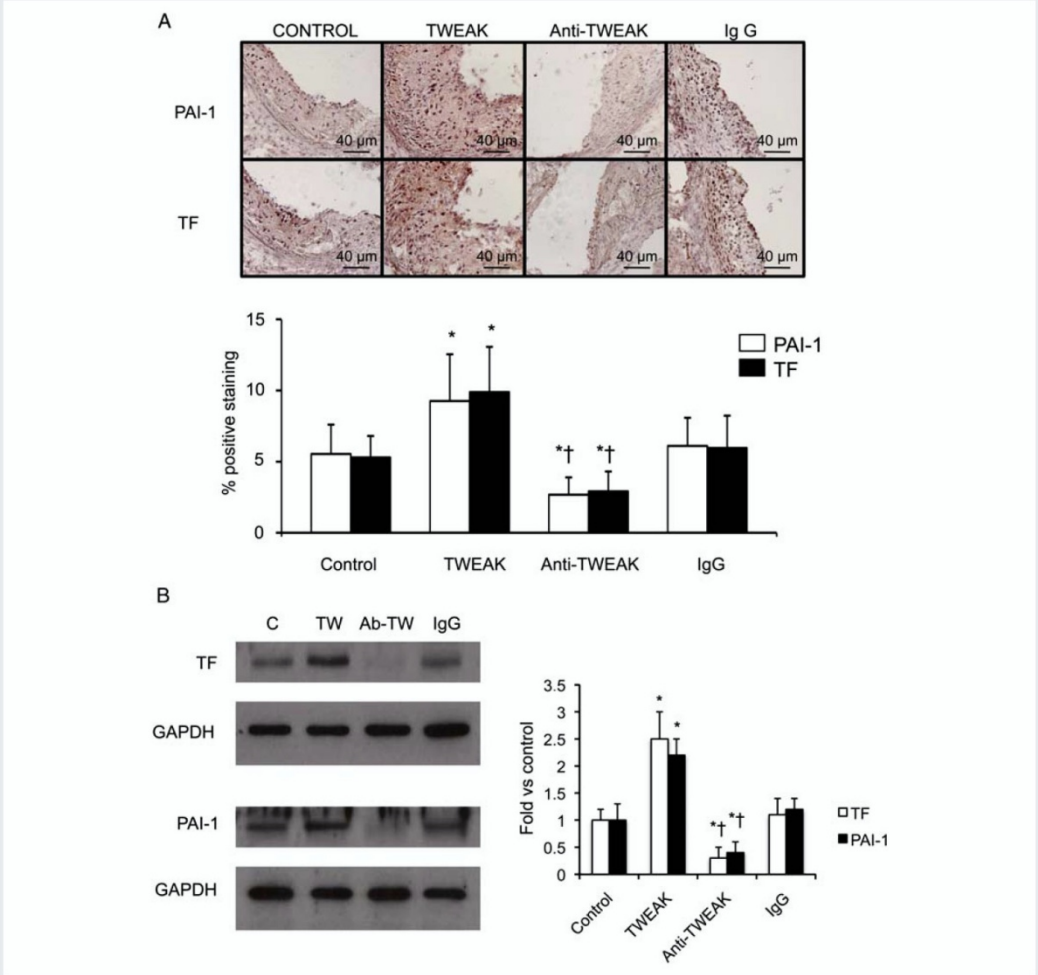


Figure 5 Effect of TWEAK and anti-TWEAK mAb on PAI-1 and TF expression in advanced atherosclerotic lesions in *ApoE* $^{-/-}$ mice. Representative immunostaining (*A*) of PAI-1 and TF in the aortic root of *ApoE* $^{-/-}$ mice, and western blot (*B*) showing PAI-1 and TF expression in the whole aorta from animals treated with saline (control), TWEAK (10 μg/kg/day), anti-TWEAK (1000 μg/kg/day), or non-specific IgG (1000 μg/kg/day) daily for 9 days. $^{*}P < 0.05$ vs. control; and $^{\dagger}P < 0.05$ vs. IgG group. Values shown are means ± SEM, $n = 6$ per group.

TWEAK–*Fn14* axis is implicated in the control of PAI-1 and TF within vessel walls. In this context, we have previously reported that *Fn14* is expressed in both macrophages and SMCs in human atherosclerotic plaques,[1] and now we have observed that TWEAK increased the expression of both proteins in cultured hASMCs, one of the major sources of these molecules within atherosclerotic plaques.[17–18] TWEAK, through its receptor, *Fn14*, increased the mRNA, protein and activity of both molecules, indicating that TWEAK mediated its effects at a transcriptional level. It is known that tissue factor gene expression by endothelial cells, SMCs and monocytes/macrophages is mediated by the activating protein-1 (AP-1) and nuclear factor-κB (NF-κB).[26] Moreover, it has also been demonstrated that activation of PAI-1 expression by different factors in endothelial cells[27] and vascular smooth muscle cells[28] is mediated by interaction of NF-κB with its promoter. Several studies have revealed that TWEAK–*Fn14* exerts many of its actions through NF-κB activation.[29–30] Thus, TWEAK increases NF-kB activation in atherosclerotic plaques present in the aortic root of *ApoE* knockout mice and in the tubular cells of an experimental model of renal damage.[24,31]

The effect observed with TWEAK incubation in cultured hASMCs is related to the presence of its receptor, *Fn14*. Use of an anti-*Fn14* blocking antibody or *Fn14* siRNA prevented the increment observed

in PAI-1 and TF expression and activity. Overall, these data indicate that *Fn14* is responsible for prothrombotic effects observed after TWEAK incubation. Although TWEAK increased PAI-1 and TF two-fold compared with unstimulated cells, different authors have reported similar results with other stimuli. For example, Chen *et al.* have reported that C-reactive protein also increases PAI-1 expression two-fold in endothelial cells.[32] In the same way, Wu *et al.* reported that C-reactive protein augmented TF expression around two-fold in vascular smooth muscle cells.[33] These data indicate that the effect observed after treatment with TWEAK is similar to that observed with other important pro-inflammatory stimuli.

Tissue factor and PAI-1 regulate thombus formation and might play a pivotal role in thrombotic complications after plaque rupture. Indeed, evidence is emerging that PAI-1 and TF may be involved in atherogenesis not only by eliciting thrombosis but also by direct actions on vascular remodelling and plaque progression or instability. Thus, both factors induce augmentation of neointimal formation,[34,35] providing a fibrin scaffold for fibrous tissue deposition and inducing proliferation of vascular smooth muscle cells.[36,37] In addition, up-regulation of TF and PAI-1 may contribute to plaque instability and risk of thrombo-embolic complications.[38] In this sense, PAI-1 and TF inhibitors have been postulated as a therapeutic strategy for prevention of vascular disease.[17,39–40] Thereby, the inhibition of TWEAK–*Fn14* signalling might contribute to attenuation of PAI-1 and TF overexpression in vessel walls, and to inhibit atherosclerosis development and thrombosis. In this context, we have observed that *ApoE*$^{-/-}$ mice injected with blocking antibodies against TWEAK showed a reduction in PAI-1 and TF expression in atherosclerotic plaques in comparison with control mice, demonstrating that an anti-TWEAK therapy might be useful to modulate both PAI-1 and TF expression in atherosclerotic plaques. In this regard, our studies also elucidate a novel mechanism whereby anti-TWEAK therapy may be a promising strategy to prevent and reduce the clinical severity of stroke.[5]

In conclusion, the present study reveals a novel role of TWEAK in vascular disease, by enhancing PAI-1 and TF expression. Although TWEAK has previously been postulated as a mediator of many of the processes involved in atherosclerosis, this is the first time that it has been related to plaque thrombogenicity. In addition, anti-TWEAK therapy could be a novel therapeutic approach to diminish procoagulant activity within atherosclerotic plaques.

Conflict of interest: Linda C. Burkly is an employee of Biogen Idec. The other authors have no conflicts of interest.

Funding

This study was supported by Ministerio de Ciencia y Tecnología (SAF 2007/60896; SAF2007/63648), Comunidad de Madrid (S2006/GEN-0247), Fondo de Investigaciones Sanitarias (Programa Miguel Servet to L.B.-C.), Instituto de Salud Carlos III (PI10/00234), Ministerio de Sanidad y Consumo, (RETICS RD06/0014/0035), Sociedad Española de Arteriosclerosis, Fundación Ramón Areces and Fundación Mutua Madrileña.

References

1. Munoz-Garcia B, Martin-Ventura JL, Martinez E, Sanchez S, Hernandez G, Ortega L *et al.* Fn14 is upregulated in cytokine-stimulated vascular smooth muscle cells and is expressed in human carotid atherosclerotic plaques: modulation by atorvastatin. *Stroke* 2006;**37**:2044–2053.
2. Meighan-Mantha RL, Hsu DK, Guo Y, Brown SA, Feng SL, Peifley KA *et al.* The mitogen-inducible *Fn14* gene encodes a type I transmembrane protein that modulates fibroblast adhesion and migration. *J Biol Chem* 1999;**274**:33166–33176.
3. Feng SL, Guo Y, Factor VM, Thorgeirsson SS, Bell DW, Testa JR *et al.* The Fn14 immediate-early response gene is induced during liver regeneration and highly expressed in both human and murine hepatocellular carcinomas. *Am J Pathol* 2000;**156**:1253–1261.
4. Wiley SR, Cassiano L, Lofton T, Davis-Smith T, Winkles JA, Lindner V *et al.* A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity* 2001;**15**:837–846.
5. Yepes M, Brown SA, Moore EG, Smith EP, Lawrence DA, Winkles JA. A soluble Fn14-Fc decoy receptor reduces infarct volume in a murine model of cerebral ischemia. *Am J Pathol* 2005;**166**:511–520.
6. Potrovita I, Zhang W, Burkly L, Hahm K, Lincecum J, Wang MZ *et al.* Tumor necrosis factor-like weak inducer of apoptosis-induced neurodegeneration. *J Neurosci* 2004;**24**:8237–8244.
7. Justo P, Sanz AB, Sanchez-Niño MD, Winkles JA, Lorz C, Egido J *et al.* Cytokine cooperation in renal tubular cell injury: the role of TWEAK. *Kidney Int* 2006;**70**:1750–1758.
8. Muñoz-García B, Moreno JA, López-Franco O, Sanz AB, Martín-Ventura JL, Blanco J *et al.* Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) enhances vascular and renal damage induced by hyperlipidemic diet in ApoE-knockout mice. *Arterioscler Thromb Vasc Biol* 2009;**29**:2061–2068.
9. Harada N, Nakayama M, Nakano H, Fukuchi Y, Yagita H, Okumura K. Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 2002;**299**:488–493.
10. Lynch CN, Wang YC, Lund JK, Chen YW, Leal JA, Wiley SR. TWEAK induces angiogenesis and proliferation of endothelial cells. *J Biol Chem* 1999;**274**:8455–8459.
11. Kim SH, Kang YJ, Kim WJ, Woo DK, Lee Y, Kim DI *et al.* TWEAK can induce pro-inflammatory cytokines and matrix metalloproteinase-9 in macrophages. *Circ J* 2004;**68**:396–399.
12. Kim SH, Lee WH, Kwon BS, Oh GT, Choi YH, Park JE. Tumor necrosis factor receptor superfamily 12 may destabilize atherosclerotic plaques by inducing matrix metalloproteinases. *Jpn Circ J* 2001;**65**:136–138.
13. Nakayama M, Kayagaki N, Yamaguchi N, Okumura K, Yagita H. Involvement of TWEAK in interferon γ-stimulated monocyte cytotoxicity. *J Exp Med* 2000;**192**:1373–1380.
14. Nakayama M, Ishidoh K, Kayagaki N, Kojima Y, Yamaguchi N, Nalano H *et al.* Multiple pathways of TWEAK-induced cell death. *J Immunol* 2002;**168**:734–743.
15. Mackman N. Role of tissue factor in hemostasis and thrombosis. *Blood Cells Mol Dis* 2006;**36**:104–107.
16. Potempa J, Korzus E, Travis J. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J Biol Chem* 1994;**269**:15957–15960.
17. Agirbasli M. Pivotal role of plasminogen-activator inhibitor 1 in vascular disease. *Int J Clin Pract* 2005;**59**:102–106.
18. Taubman MB, Fallon JT, Schecter AD, Giesen P, Mendlowitz M, Fyfe BS *et al.* Tissue factor in the pathogenesis of atherosclerosis. *Thromb Haemost* 1997;**78**:200–204.
19. Moreno PR, Bernardi VH, López-Cuéllar J, Murcia AM, Palacios IF, Gold HK *et al.* Macrophages, smooth muscle cells, and tissue factor in unstable angina. Implications for cell-mediated thrombogenicity in acute coronary syndromes. *Circulation* 1996;**94**:3090–3097.
20. Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT *et al.* Release of active tissue factor by human arterial smooth muscle cells. *Circ Res* 2000;**87**:126–132.
21. Toschi V, Gallo R, Lettino M, Fallon JT, Gertz SD, Fernández-Ortiz A *et al.* Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* 1997;**95**:594–599.
22. Lupu F, Bergonzelli GE, Heim DA, Cousin E, Genton CY, Bachmann F *et al.* Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. *Arterioscler Thromb* 1993;**13**:1090–1100.
23. Blanco-Colio LM, Muñoz-García B, Martín-Ventura JL, Lorz C, Díaz C, Hernández G *et al.* 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors decrease Fas ligand expression and cytotoxicity in activated human T lymphocytes. *Circulation* 2003;**108**:1506–1513.
24. Reilly CF, McFall RC. Platelet-derived growth factor and transforming growth factor-β regulate plasminogen activator inhibitor-1 synthesis in vascular smooth muscle cells. *J Biol Chem* 1991;**266**:9419–9427.
25. Schneider DJ, Ricci MA, Taatjes DJ, Baumann PQ, Reese JC, Leavitt BJ *et al.* Changes in arterial expression of fibrinolytic system proteins in atherogenesis. *Arterioscler Thromb Vasc Biol* 1997;**17**:3294–3301.
26. Mackman N. Regulation of the tissue factor gene. *FASEB J* 1995;**9**:883–889.
27. Swiatkowska M, Szemraj J, Cierniewski CS. Induction of PAI-1 expression by tumor necrosis factor α in endothelial cells is mediated by its responsive element located in the 4G/5G site. *FEBS J* 2005;**272**:5821–5831.
28. Diebold I, Djordjevic T, Hess J, Görlach A. Rac-1 promotes pulmonary artery smooth muscle cell proliferation by upregulation of plasminogen activator inhibitor-1: role of NFκB-dependent hypoxia-inducible factor-1α transcription. *Thromb Haemost* 2008;**100**:1021–1028.

29. Li H, Mittal A, Paul PK, Kumar M, Srivastava DS, Tyagi SC *et al*. Tumor necrosis factor-related weak inducer of apoptosis augments matrix metalloproteinase 9 (MMP-9) production in skeletal muscle through the activation of nuclear factor-κB-inducing kinase and p38 mitogen-activated protein kinase: a potential role of MMP-9 in myopathy. *J Biol Chem* 2009;**284**:4439–4450.
30. Dogra C, Changotra H, Mohan S, Kumar A. Tumor necrosis factor-like weak inducer of apoptosis inhibits skeletal myogenesis through sustained activation of nuclear factor-κB and degradation of MyoD protein. *J Biol Chem* 2006;**281**:10327–10336.
31. Sanz AB, Justo P, Sanchez-Niño MD, Blanco-Colio LM, Winkles JA, Kreztler M *et al*. The cytokine TWEAK modulates renal tubulointerstitial inflammation. *J Am Soc Nephrol* 2008;**19**:695–703.
32. Chen C, Nan B, Lin P, Yao Q. C-reactive protein increases plasminogen activator inhibitor-1 expression in human endothelial cells. *Thromb Res* 2008;**122**:125–133.
33. Wu J, Stevenson MJ, Brown JM, Grunz EA, Strawn TL, Fay PW. C-reactive protein enhances tissue factor expression by vascular smooth muscle cells. Mechanisms and in vivo significance. *Arterioscler Thromb Vasc Biol* 2008;**28**:698–704.
34. Zhu Y, Farrehi PM, Fay WP. Plasminogen activator inhibitor type 1 enhances neointima formation after oxidative vascular injury in atherosclerosis-prone mice. *Circulation* 2001;**103**:3105–3110.
35. Singh R, Pan S, Mueske CS, Witt T, Kleppe LS, Peterson TE *et al*. Role for tissue factor pathway in murine model of vascular remodeling. *Circ Res* 2001;**89**:71–76.
36. Chen Y, Budd RC, Kelm RJ Jr, Sobel BE, Schneider DJ. Augmentation of proliferation of vascular smooth muscle cells by plasminogen activator inhibitor type 1. *Arterioscler Thromb Vasc Biol* 2006;**26**:1777–1783.
37. Gasic GP, Arenas CP, Gasic TB, Gasic GJ. Coagulation factors X, Xa, and protein S as potent mitogens of cultured aortic smooth muscle cells. *Proc Natl Acad Sci USA* 1992; **89**:2317–2320.
38. Sayed S, Cockerill GW, Torsney E, Poston R, Thompson MM, Loftus IM. Elevated tissue expression of thrombomodulatory factors correlates with acute symptomatic carotid plaque phenotype. *Eur J Vasc Endovasc Surg* 2009;**38**: 20–25.
39. Stephens AC, Rivers RP. Suppression of human monocyte tissue factor synthesis by antisense oligodeoxynucleotide. *Thromb Res* 1997;**85**:387–398.
40. Morrow DA, Murphy SA, McCabe CH, Mackman N, Wong HC, Antman EM. Potent inhibition of thrombin with a monoclonal antibody against tissue factor (Sunol-cH36): results of the PROXIMATE-TIMI 27 trial. *Eur Heart J* 2005;**26**: 682–688.

Atherosclerosis 212 (2010) 333–338

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAAs). A potential link of oxidative stress with AAA evolution

R. Martinez-Pinna [a], J.S. Lindholt [b], L.M. Blanco-Colio [a], T. Dejouvencel [c], J. Madrigal-Matute [a], P. Ramos-Mozo [a], M. Vega de Ceniga [d], J.B. Michel [c], J. Egido [a], O. Meilhac [c], J.L. Martin-Ventura [a,*]

[a] IIS-Vascular Research Lab, Fundación Jiménez Diaz, Madrid, Spain
[b] Vascular Research Unit, Viborg Hospital, Denmark
[c] Inserm, U698, Univ Paris 7, CHU X-Bichat, Paris, France
[d] Vascular Surgery, Hospital Galdakao, Bilbao, Spain

ARTICLE INFO

Article history:
Received 9 January 2010
Received in revised form 11 May 2010
Accepted 19 May 2010
Available online 1 June 2010

Keywords:
Biomarkers
Abdominal aortic aneurysm
Oxidative stress

ABSTRACT

Objective: Oxidative stress is a main mechanism involved in vascular pathologies. Increased thioredoxin (TRX) levels have been observed in several oxidative stress-associated cardiovascular diseases. We aim to test the potential role of TRX as a biomarker of oxidative stress in abdominal aortic aneurysm (AAA).
Methods: TRX levels were analysed in both AAA intraluminal thrombus (ILT) tissue and in tissue-conditioned media by immunohistochemistry, Western blot and ELISA. Moreover, serum TRX levels were assessed in AAA Caucasian patients by ELISA.
Results: TRX was mainly localized in the luminal part of ILT in AAA. Compared with the abluminal layer, TRX release was increased in the luminal layer of the ILT of AAA (31 ± 9 ng/ml vs. 9 ± 3 ng/ml, $p < 0.05$). The interest of this approach is that we can identify proteins potentially released into the blood compartment, which could serve as biomarkers of the pathology. In a training population, serum TRX levels were significantly increased in patients with AAA relative to healthy subjects (50 ± 6 ng/ml vs. 26 ± 3 ng/ml, $p < 0.05$). These results were validated in a second independent group of patients. Moreover, a positive correlation between TRX and AAA size (rho = 0.5, $p < 0.001$) was observed. Finally, in AAA samples with follow-up, TRX was positively associated to aneurismal growth rate (rho = 0.25, $p = 0.027$).
Conclusions: TRX release is increased in the luminal part of AAA and TRX serum levels are increased in AAA patients compared with healthy subjects. TRX levels correlates with AAA size and expansion, suggesting its potential role as a biomarker of AAA evolution.

1. Introduction

Abdominal aortic aneurysm (AAA) is an important health problem in elderly. In cross-sectional studies the prevalence varies from 3% to 8%. In elderly men AAA may cause as much as 2–3% of all deaths. The incidence of asymptomatic and ruptured AAA has increased during the last decades [1].

Efforts to limit the mortality rate from AAA rupture depend on early detection and elective AAA repair. The indication for elective repair preventing rupture is mainly based upon the maximal diameter of AAA above 5 or 5.5 cm. The benefit of early detection of AAAs by ultrasound screening is limited because most AAAs are too small to operate at the time of diagnosis, early repair of small AAA has been demonstrated to be inefficient and there is currently no established treatment for small AAAs [2,3]. Most AAAs shows discontinuous growth patterns and alternate periods of stability and non-growth with periods of acute expansion and occasionally rupture. In this respect, the diameter of the AAA is a surrogate marker of the growth rate, which reflects the magnitude of the degenerative process of the wall, and thus becomes a surrogate marker of rupture. Consequently, biomarkers of size, growth and rupture could give us a more nuanced indication for surgery. Moreover, identification of such biomarkers could also afford novel pathogenic pathways and may thus open possibilities for pharmacological inhibition of growth, providing tools for monitoring this inhibition [4].

AAA are characterized by the presence of a mural hemothrombus containing non-cellular and cellular blood components including platelets, red blood cells (RBCs) and polymorphonuclear neutrophils (PMN) which represent the major class of leukocytes,

Abbreviations: TRX, thioredoxin; AAA, abdominal aortic aneurysm; RBCs, red blood cells; PMNs, polymorphonuclear neutrophils; MPO, myeloperoxidase; MIF, macrophage inhibitory factor.

* Corresponding author at: Vascular Research Lab, Fundación Jiménez Diaz, Autonoma University, Av. Reyes Católicos 2, 28040 Madrid, Spain.
Tel.: +34 915504800; fax: +34 915442636.
E-mail address: jlmartin@fjd.es (J.L. Martin-Ventura).

0021-9150/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2010.05.031

particularly abundant within the luminal layer of the thrombus. Upon activation, PMN release proteases but also myeloperoxidase (MPO) causing oxidative damages. The combination of NADPH oxidase, NO synthase and MPO leads to the production of a variety of reactive oxygen species such as superoxide anion, hydroxyl radicals or peroxynitrite. In addition, the trapping of RBCs within the thrombus may lead to hemolysis and subsequent release of the pro-oxidant hemoglobin, in particular able to generate OH° from H_2O_2 by the Fenton reaction. This biological activity of the luminal layer of the thrombus is therefore a source of potential biomarkers coming from activated or dying PMNs and RBCs. Thioredoxin (TRX) is an important intracellular antioxidant enzyme that is elevated in plasma of patients with coronary atherosclerosis [5,6] and associated with the presence of intraplaque hemorrhage [7]. In the present work, we sought for the presence of TRX in the AAA thrombus, which could be released into the blood compartment. We thus assessed TRX in conditioned media from the different layers of AAA thrombus, and then in the serum of patients with AAA.

2. Materials and methods

2.1. AAA tissue and tissue-conditioned media

Ten AAA thrombus samples were collected during surgical repair and dissected into luminal and abluminal parts (respectively at the interface with circulating blood and with the remaining media). AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol (REflet Sanguin de l'évolutivité des Anévrysmes de l'Aorte abdominale, CCPPRB Paris-Cochin n° 2095, n° 1930 and n° 1931) [8]. All patients gave their informed written consent, and the protocol was approved by a French ethics committee (CCPPRB, Cochin Hospital). Luminal and abluminal layers of AAA thrombus were cut into small pieces (5 mm^2) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 h at 37 °C (6 ml/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation as 3000 × *g* for 10 min at 20 °C. Protein concentration of each conditioned medium was measured using the Bradford assay (Bio-Rad).

2.2. AAA serum

2.2.1. Spanish cohort study

The study was approved by Spanish Center's Research and Ethics Committees, and informed consent from the patients and the controls for their inclusion in the study was obtained.

88 consecutive patients (Table 1 *online*) with an asymptomatic infrarenal AAA were recruited and the AAA size at the time of blood sample collection was registered. We excluded patients with symptomatic or inflammatory AAA, multiple synchronic aneurysms (thoracic, femoral, popliteal), and AAA with a location other than infrarenal. We also excluded patients with active inflammatory or acute infectious processes, surgical procedures or major trauma in the previous 60 days, and chronic antinflammatory or immunosupressive medication.

30 controls were recruited from a screening program that is currently being performed among the population in the area under our care. They were randomly selected from the screened individuals with non-dilated (<30 mm, confirmed with abdominal ultrasound) infrarenal aortas (Table 1 *online*).

2.2.2. The Danish cohort study

In 1994, half (4404) of all 65–73-year-old males in Viborg County, Denmark, were invited to B-mode-ultrasonographic screening for AAA at their regional hospital. The trial was approved by the respective local scientific ethics committees and reported to the Danish Central Control of Registers.

An AAA was defined as an infrarenal aortic diameter of 30 mm or more, and AAAs > 50 mm were referred for surgery. AAAs (30–49 mm) were offered yearly follow-up examinations to check for any expansion [9]. Two observers were used. Their arimetric interobserver variation (2SD) of the measurements was 1.4 mm [10]. Serum samples were stored in multiple aliquots at −70 °C until analysis. A random sample of 78 cases was used in this study (Table 2 *online*). The expansion rate was calculated as the change in the anteriorposterior (AP) diameter during the whole observation period, transformed to annual units.

2.3. Western blot

Equal amounts (5 μl) of conditioned medium (previously normalized to tissue weight: 1 g/6 ml) were electrophoresed as described [11]. Membranes were incubated with anti-thioredoxin antibody (Abcam, Rabbit polyclonal Ab16835, used at 0.5 μg/ml), washed with TBS-T, and incubated with anti-rabbit horseradish peroxidase antibody (1:1500). After five washes, the signal was detected using a chemiluminescence kit.

2.4. ELISA

We quantified with commercial kits the soluble concentrations of TRX (Redox Bioscience) in both conditioned media and serum, following the manufacturer's instructions. The interassay and intraassay variabilities for TRX were respectively 15% and 6%.

2.5. Immunohistochemistry

AAA thrombus samples were fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5 μm sections, using anti-thioredoxin Ab16835 (5 μg/ml) as primary antibody. Peroxidase LSAB Dako kit (Dako) followed by Histogreen substrate (AbCys SA) was used for detection. Sections were then counterstained with Nuclear Red. Control irrelevant immunoglobulins (Dako) were applied at the same concentrations as primary antibodies to assess nonspecific staining.

2.6. Statistics

Results are expressed as mean ± SEM. The Wilcoxon paired test was used to analyse differences in TRX levels between luminal and abluminal supernatants of the same samples. The analysis between small and large AAA and control groups was performed with non-parametric tests (Mann–Whitney *U* tests). Spearman's rho correlation analyses were used to examine an association between TRX, MPO, maximal AAA-diameter and annual expansion rate. The receiving-operating characteristic (ROC) curve analysis was performed to test the predictive clinical value of TRX to discriminate between both, small and large AAA (< or >5–5.5 cm) and with those small AAA with low/high risk evolution (< or >2 mm/year, mean annual growth). For the analysis of the ROC curve, the null hypothesis was that the test had a performance similar to the diagonal line, i.e., the area under the curve was 0.5. If the lowest 95% confidence limit for the area under the curve was more than 0.5, a significant predictive test was said to be present.

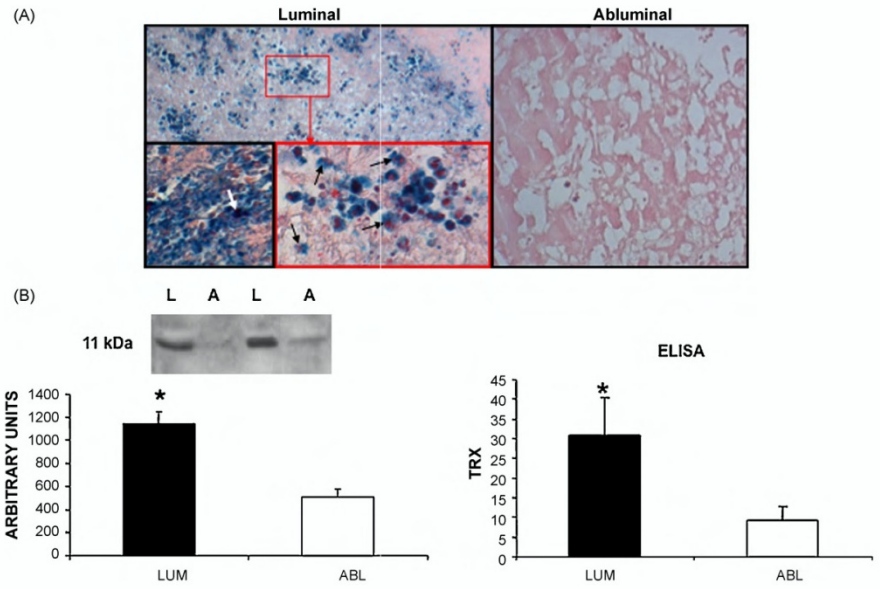


Fig. 1. Thioredoxin is produced and released by the luminal part of AAA thrombus. (A) Immunostaining of TRX on the luminal and abluminal layers of the AAA thrombus (10×, black inset: 20×, red inset: 40×). Positive staining appears in green. The red inset shows positive cells, mostly neutrophils with their characteristic poly-lobed nuclei. Some of them are releasing their intracellular content (arrows). Black inset: freshly formed luminal thrombus showing positive erythrocytes (white arrow: poly-lobed nucleus). (B) Western blot and ELISA of anti-TRX on conditioned medium from luminal (L) and abluminal (A) part of the mural thrombus ($n = 10$, $^*p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Experimental results

3.1. TRX is mainly localized and released by the luminal part of AAA thrombus

We first analysed the presence and localization of TRX in human AAA thrombus. As shown in Fig. 1A, the luminal part of the thrombus shows an important staining associated with poly-lobed nuclei cells, likely to be neutrophils. In some areas, corresponding to most recently formed thrombus, fibrin and erythrocytes show an intense positivity, which may also participate in TRX release by the luminal part of the thrombus. Since TRX is released from cells in response to oxidative stress, we analysed the levels of TRX in the conditioned media of the different layers of the thrombus in human AAA samples. Compared with the abluminal layer, TRX release was increased in the luminal layer of the ILT of AAA, as assessed by Western blot and confirmed by ELISA (31 ± 9 ng/ml vs. 9 ± 3 ng/ml, $p < 0.001$, Fig. 1B and C). *In vitro* experiments using PMN isolated from blood showed that they are able to release large amount of TRX after activation by fMLP, as assessed by Western blot of the resulting supernatant (data not shown). This suggests that neutrophil degranulation may participate in TRX release by the luminal layer of ILT samples.

3.2. Increased TRX serum levels in AAA patients

In a training population, we have shown that circulating TRX levels are significantly increased in the serum of patients with AAA ($n = 30$) relative to control ($n = 15$) subjects (50 ± 6 ng/ml vs. 26 ± 3 ng/ml, $p < 0.05$). We validated the results obtained in a second independent group of small ($n = 34$) and large AAA ($n = 24$) patients and new controls ($n = 15$) from a multicentre study. As observed in Fig. 2, we found that patients with large AAA have significantly increased TRX levels as compared to both small AAA and controls (77 ± 11 ng/ml vs. 38 ± 3 ng/ml and 42 ± 4 ng/ml, $p < 0.05$ for both). When TRX levels were analysed by subgroups depending

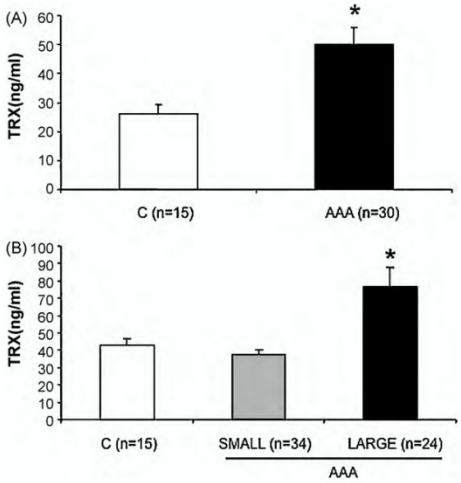


Fig. 2. TRX levels in AAA. (A) TRX levels are significantly increased in serum of patients with AAA ($n = 30$) vs. controls ($n = 15$) ($^*p < 0.05$). (B) TRX levels are significantly increased in serum of patients with large AAA ($n = 24$) in relation to small AAA ($n = 34$) and controls ($n = 15$) ($^*p < 0.05$).

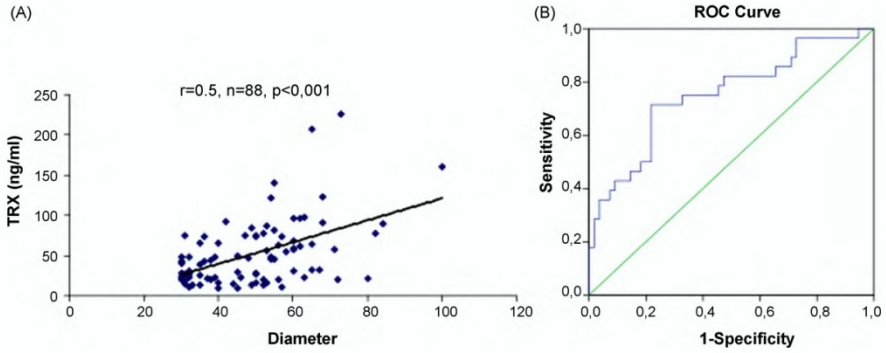


Fig. 3. Correlation of TRX with AAA size. (A) Correlation between TRX and AAA size in the Spanish cohort study (Spearman's rho = 0.5, $p < 0.001$, $n = 88$ patients). (B) ROC curve analysis to predict patients with small or large AAA depending on TRX levels.

on different risk factors (hypertension, dyslipidemia, smoking), no significant differences were observed.

3.3. TRX correlates with AAA-diameter and growth rate

Since AAA diameter is a surrogate marker of the growth rate, we studied its potential correlation with TRX. Interestingly, TRX levels and AAA diameter show a significant positive correlation ($r = 0.5$, $p < 0.001$, Fig. 3). Interestingly, TRX was able to discriminate between patients with small and large AAA (area under ROC curve = 0.75, 95% CI, 0.64–0.87, $p < 0.001$), with an optimal cutpoint of 50 ng/ml associated with a sensitivity of 71.4%, and a specificity of 76.4%.

In the Viborg study, Spearman's correlation coefficient between TRX, AAA-size and AAA-growth rate was 0.15 ($p = 0.19$) and 0.25 ($p = 0.03$, Fig. 4). Interestingly, TRX also correlated with macrophage inhibitory factor (MIF) levels ($r = 0.5$, $p < 0.001$). Furthermore, TRX levels were lower in those patients with low aspirin treatment (35.6 ± 7.2 ng/ml vs. 53.9 ± 5.7 ng/ml, $p < 0.05$). Interestingly, TRX was predictive of patients expanding >2 mm/year (area under ROC curve = 0.67, 95% CI, 0.55–0.79, $p = 0.01$), with an optimal cutpoint of 30 ng/ml associated with a 62% sensitivity and specificity both.

4. Discussion

AAA result from atherothrombotic complications in an infrarenal segment of the aorta, leading to a permanent arterial dilatation associated with proteolytic injury of the media. In contrast to stenosing evolution of atherothrombosis such as in carotid, femoral or coronary arteries, AAA presents an outward progression due to the weakening of the media as a consequence of extracellular matrix proteolysis and smooth muscle cell rarefaction. The formation of a luminal thrombus may be considered as a compensatory mechanism in response to flow perturbations associated with the dilatation. The mural thrombus is continuously self-forming at its luminal pole and subjected to proteolysis at the interface with the residual media [12]. The quasi-absence of macrophages within the thrombus may be associated with an impaired cicatrisation, potentially due to an overwhelming proteolytic and oxidative environment [13]. Neutrophils are the major class of leukocytes present/recruited in the luminal thrombus. Their short-half life suggests that they are rapidly activated, subsequently undergo apoptosis and then post-apoptotic necrosis. The release of PMN intracellular content associated with erythrocyte lysis may lead to a tremendous pro-oxidant environment within the thrombus. In the present study, immunohistochemical analysis showed that both RBCs and PMNs exhibited positive staining for

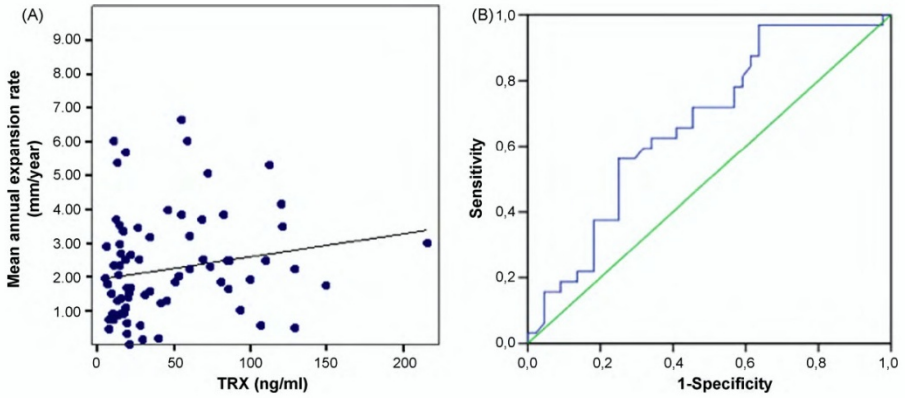


Fig. 4. Correlation of TRX with AAA growth. The correlation between serum TRX levels and aneurysmal growth rate among prospectively followed AAA sized 3–5 cm in maximal AAA diameter in the Viborg cohort study. (B) ROC curve analysis for predicting cases expanding >2 mm/year related to TRX levels.

TRX, particularly at the site of the freshly formed thrombus. Interestingly, RBCs contain TRX [14] and it has recently been observed that TRX upregulation in response to increased oxidative stress was associated with intraplaque hemorrhage of coronary culprit lesions, suggesting its potential role as a marker of plaque instability [7].

Oxidative stress is increased in patients with AAA. Plasma levels of malondialdehyde, the major endproduct of lipid oxidation, were significantly increased in AAA patients vs. controls with a positive correlation with the AAA diameter [15]. Superoxide anions, as well as lipid peroxidation products were assessed in AAA arterial wall samples as compared with adjacent non-abdominal segments, showing an increased oxidative stress and associated derived-products in AAA segments [16]. Lipid peroxidation was also increased in aortic dissection media relative to control samples [17]. However, as far as we know, no studies were performed on the mural hemo-thrombus as a potential source of oxidative stress biomarkers. TRX is released from cells in response to oxidative stress [18]. In this respect, we have observed increased TRX levels in the conditioned medium of the luminal part of the thrombus, which is rich in activated PMNs and RBCs. We hypothesized that the plasma compartment could reflect what was observed in the arterial conditioned medium and thus, proteins potentially released to the blood could serve as biomarkers of the pathology. We show here for the first time that TRX levels were significantly increased in serum of patients with AAA in relation to controls. These were validated in two additional analyses with samples from a multicentric study.

Increased circulating TRX levels have been observed in conditions of oxidative stress and inflammation, among them cardiovascular diseases. Thioredoxin levels are elevated in patients with AMI, and higher TRX levels predicted subsequent failure of coronary reperfusion therapy [19]. In these patients, increased blood TRX was associated with platelet hyperaggregability and lower left ventricular ejection fraction [6]. Moreover, TRX levels were significantly increased in patients with unstable angina and were associated with recurrent myocardial ischemia [5]. Similarly, increased MPO levels have been reported in patients with cardiovascular diseases [20]. We have also observed a significant correlation between TRX and MPO in blood of AAA patients (unpublished observation), suggesting their potential association *in vivo*. Since both MPO and TRX are released by activated PMNs and we observed TRX expression by PMNs in AAA, it seems plausible that activated PMNs could be a source of extracellular TRX, in conjunction with RBCs.

AAA diameter is a surrogate marker of the growth rate and is the clinical parameter used to the management of AAA patients, thus we studied its potential correlation with MPO and TRX. A modest correlation between MPO and AAA diameter was observed (unpublished observation). Interestingly, TRX levels and AAA diameter show a significant positive correlation. As mentioned in the introduction, we urgently need biomarkers of AAA. However, one has to keep in mind that management of AAA only includes inhibition of further expansion and rupture but data and biomaterials for rupture are very difficult to achieve due to preventive repair of large AAA. Instead, surrogate markers are used; AAA-size and AAA-growth rate. Clearly, prospective data concerning growth rate holds the highest level of evidence (level II) as it reflects the present catabolism of the Aorta, while AAA size may reflect aggressive catabolism in the past, but presently stable conditions. Studies on AAA-size cover the whole range of AAA, and are in addition easy and inexpensive to gain compared to long-term cohort studies on growth rate. In all, the two surrogate markers must be considered to be complementary. In this study, TRX correlates significantly with both AAA size and AAA growth from different populations, and combined with the observation of a similar median concentration in small AAA in the two populations suggest the findings are generalisable. However, the correlation with AAA size was not statistically significant in the Viborg cohort study, but it may be due to the fact that only small AAA was included.

Although these data suggest that TRX could reflect a response to oxidative stress in various diseases, the functional consequences of TRX upregulation are not completely understood. The potential biological significance of TRX upregulation can be inferred from experimental studies, which support a role as an antioxidant and anti-inflammatory protein. TRX could modulate NADPH oxidase-mediated generation of superoxide anion by interacting with p40Phox [21]. In addition, it has been shown that TRX could modulate heme-oxygenase 1 (HO-1)[22]. Interestingly, we have observed that TRX and HO-1 levels correlates in AAA conditioned media (unpublished observations). Extracellular TRX has been reported to reduce interleukin 1 beta expression by monocyte–macrophages in inflammatory conditions [23]. More recently, it has been suggested that the anti-inflammatory mechanisms of TRX could be mediated, at least in part, by MIF downregulation [24]. In this respect, serum-MIF levels correlated significantly with annual AAA expansion rate and initial AAA size [25]. In the same samples from the Viborg Cohort study, we have observed a significant strong positive correlation between TRX and MIF. In addition, circulating thioredoxin suppresses neutrophil chemotaxis [26]. Moreover, it has been shown that TRX is upregulated during PMN apoptosis, which is crucial for resolution of inflammation. Because neutrophil extravasation to the AAA enhance the growing and potential rupture of AAA, it seems plausible that increased TRX levels in patients with AAA is the biological response of the organism to prevent such excessive neutrophil accumulation within the intraluminal thrombus.

Consistent with this, TRX could be a potential therapeutic target in cardiovascular disorders. In this respect, administration of recombinant TRX prevented myosin-induced myocarditis [27] and smoke-induced emphysema [28] in animal models. Interestingly, we have observed that TRX levels were lower in those patients with low aspirin treatment. In this respect, it has been already shown that increased plasma thioredoxin levels were associated with platelet hyperaggregability [6]. We have previously demonstrated that AAA growth is inhibited by antiplatelets in rats [29] and we have reported that low dose aspirin in AAA 4–5 cm is associated with lower growth rate in humans [30].

In the present study, serum thioredoxin levels are associated with the presence and progression of AAA suggesting its potential role as a biomarker of AAA evolution. Further prospective studies in large patients cohorts with different ethnic backgrounds are needed to confirm these results.

Acknowledgements

The paper have been supported by the European Community, FAD project (FP-7, HEALTH F2-2008-200647), the Spanish Ministerio de Ciencia y Tecnología (SAF2007/63648), (SAF2010-21852, SAF 2007-60896) HF2007-0120, Fundacion Ramon Areces, CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035) (Programa Miguel Servet to L.M.B.-C.) and EUS2008-03565. TJ was supported by grants from the Nouvelle Société Française d'Athérosclérose and Fondation pour la Recherche Médicale. We want to thank Dr. Cesar Aparicio and Dr. Antonio Martin-Conejero for the collection of human samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.05.031.

References

[1] Lindholt JS, Jensen LP. Vascular surgical procedures in Denmark 1996–2004 (Karkirurgiske procedurer i Danmark 1996–2004). Ugeskr Laeger 2007;169:4066–70.
[2] Powell JT, Brown LC, Forbes JF, et al. Final 12-year follow-up of surgery versus surveillance in the UK Small Aneurysm Trial. Br J Surg 2007;94:702–8.
[3] Baxter BT, Terrin MC, Dalman RL. Medical management of small abdominal aortic aneurysms. Circulation 2008;117:1883–9.
[4] Golledge J, Tsao PS, Dalman RL, Norman PE. Circulating markers of abdominal aortic aneurysm presence and progression. Circulation 2008;118:2382–92.
[5] Hokamaki J, Kawano H, Soejima H, et al. Plasma thioredoxin levels in patients with unstable angina. Int J Cardiol 2005;99:225–31.
[6] Miyamoto S, Sakamoto T, Soejima H, et al. Plasma thioredoxin levels and platelet aggregability in patients with acute myocardial infarction. Am Heart J 2003;146:465–71.
[7] Nishihira K, Yamashita A, Imamura T, et al. Thioredoxin in coronary culprit lesions: possible relationship to oxidative stress and intraplaque hemorrhage. Atherosclerosis 2008;201:360–7.
[8] Caligiuri G, Rossignol P, Julia P, et al. Reduced immunoregulatory CD31+ T cells in patients with atherosclerotic abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 2006;26:618–23.
[9] Lindholt JS, Juul S, Fasting H, Henneberg EW. Screening for abdominal aortic aneurysms: single centre randomised controlled trial. BMJ 2005;330:750.
[10] Lindholt JS, Vammen S, Juul S, Henneberg EW, Fasting H. The validity of ultrasonographic scanning as screening method for abdominal aortic aneurysm. Eur J Vasc Endovasc Surg 1999;17:472–5.
[11] Martin-Ventura JL, Leclercq A, Blanco-Colio LM, et al. Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. Atherosclerosis 2007;194:334–41.
[12] Michel JB. Contrasting outcomes of atheroma evolution: intimal accumulation versus medial destruction. Arterioscler Thromb Vasc Biol 2001;21:1389–92.
[13] Fontaine V, Touat Z, Mtairag el M, et al. Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus. Am J Pathol 2004;164:2077–87.
[14] Cha MK, Kim IH. Thioredoxin-linked peroxidase from human red blood cell: evidence for the existence of thioredoxin and thioredoxin reductase in human red blood cell. Biochem Biophys Res Commun 1995;217:900–7.
[15] Papalambros E, Sigala F, Georgopoulos S, et al. Malondialdehyde as an indicator of oxidative stress during abdominal aortic aneurysm repair. Angiology 2007;58:477–82.
[16] Miller Jr FJ, Sharp WJ, Fang X, et al. Oxidative stress in human abdominal aortic aneurysms: a potential mediator of aneurysmal remodeling. Arterioscler Thromb Vasc Biol 2002;22:560–5.
[17] Liao M, Liu Z, Bao J, et al. A proteomic study of the aortic media in human thoracic aortic dissection: implication for oxidative stress. J Thorac Cardiovasc Surg 2008;136:65–72, 72.e1–3.
[18] Rubartelli A, Bajetto A, Allavena G, Wollman E, Sitia R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. J Biol Chem 1992;267:24161–4.
[19] Soejima H, Suefuji H, Miyamoto S, et al. Increased plasma thioredoxin in patients with acute myocardial infarction. Clin Cardiol 2003;26:583–7.
[20] Zhang R, Brennan ML, Fu X, et al. Association between myeloperoxidase levels and risk of coronary artery disease. JAMA 2001;286:2136–42.
[21] Nishiyama A, Ohno T, Iwata S, et al. Demonstration of the interaction of thioredoxin with p40phox, a phagocyte oxidase component, using a yeast two-hybrid system. Immunol Lett 1999;68:155–9.
[22] Trigona WL, Mullarky IK, Cao Y, Sordillo LM. Thioredoxin reductase regulates the induction of hemo oxygenase-1 expression in aortic endothelial cells. Biochem J 2006;394(Pt 1):207–16.
[23] Billiet L, Furman C, Larigauderie G, et al. Extracellular human thioredoxin-1 inhibits lipopolysaccharide-induced interleukin-1beta expression in human monocyte-derived macrophages. J Biol Chem 2005;280:40310–8.
[24] Tamaki H, Nakamura H, Nishio A, et al. Human thioredoxin-1 ameliorates experimental murine colitis in association with suppressed macrophage inhibitory factor production. Gastroenterology 2006;131:1110–21.
[25] Pan JH, Lindholt JS, Sukhova GK, et al. Macrophage migration inhibitory factor is associated with aneurysmal expansion. J Vasc Surg 2003;37:628–35.
[26] Nakamura H, Herzenberg LA, Bai J, et al. Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. Proc Natl Acad Sci USA 2001;98:15143–8.
[27] Liu W, Nakamura H, Shioji K, et al. Thioredoxin-1 ameliorates myosin-induced autoimmune myocarditis by suppressing chemokine expressions and leukocyte chemotaxis in mice. Circulation 2004;110:1276–83.
[28] Sato A, Hoshino Y, Hara T, et al. Thioredoxin-1 ameliorates cigarette smoke-induced lung inflammation and emphysema in mice. J Pharmacol Exp Ther 2008;325:380–8.
[29] Dai J, Louedec L, Philippe M, Michel JB, Houard X. Effect of blocking platelet activation with AZD6140 on development of abdominal aortic aneurysm in a rat aneurysmal model. J Vasc Surg 2009;49:719–27.
[30] Lindholt JS, Sorensen HT, Michel JB, Thomsen HF, Henneberg EW. Low-dose aspirin may prevent growth and later surgical repair of medium-sized abdominal aortic aneurysms. Vasc Endovascular Surg 2008;42:329–34.

PUESTA AL DÍA

Biomarcadores en la medicina cardiovascular

José L. Martín-Ventura[a], Luis M. Blanco-Colio[a], José Tuñón[b], Begoña Muñoz-García[a], Julio Madrigal-Matute[a], Juan A. Moreno[a], Melina Vega de Céniga[c] y Jesús Egido[a]

[a]Laboratorio de Patología Vascular. Fundación Jiménez Díaz. Universidad Autónoma de Madrid. Madrid. España.
[b]Departamento de Cardiología. Fundación Jiménez Díaz. Universidad Autónoma de Madrid. Madrid. España.
[c]Departmento de Angiología y Cirugía Vascular. Hospital de Galdakao-Usansolo. Galdácano. Vizcaya. España.

Las enfermedades cardiovasculares son la primera causa de muerte en el mundo occidental. El proceso patológico que subyace a ellas es un engrosamiento de la pared arterial debido a la formación de placas ateroscleróticas, las cuales se complican frecuentemente con un trombo y pueden dar lugar a síndrome coronario agudo o accidente cerebrovascular. Uno de los mayores retos de la medicina cardiovascular es encontrar la manera de predecir el riesgo de un sujeto de sufrir un evento trombótico agudo.

En las últimas décadas, hay un gran interés en la búsqueda de biomarcadores diagnósticos y pronósticos que puedan ser identificados en sangre. Entre ellos, la proteína C reactiva es la más conocida. Otros, como el ligando de CD40 soluble, pueden predecir eventos cardiovasculares. En cambio, hasta el momento no hay un biomarcador aceptado en la práctica clínica. Actualmente, existen diversas técnicas de alto rendimiento como la proteómica, que permite la detección de múltiples biomarcadores potenciales. Estas aproximaciones pueden identificar en un futuro próximo nuevos biomarcadores que, junto con las técnicas de imagen, pueden ayudar a mejorar la predicción de eventos vasculares agudos.

Palabras clave: *Biomarcadores. Aterotrombosis. Proteómica.*

Biomarkers in Cardiovascular Medicine

Cardiovascular disease is the principal cause of death in developed countries. The underlying pathological process is arterial wall thickening due to the formation of atherosclerotic plaque, which is frequently complicated by thrombus, thereby giving rise to the possibility of acute coronary syndrome or stroke. One of the major challenges in cardiovascular medicine is to find a way of predicting the risk that an individual will suffer an acute thrombotic event. During the last few decades, there has been considerable interest in finding diagnostic and prognostic biomarkers that can be detected in blood. Of these, C-reactive protein is the best known. Others, such as the soluble CD40 ligand, can be used to predict cardiovascular events. However, to date, no biomarker has been generally accepted for use in clinical practice. At present, there are a number of high-performance techniques, such as proteomics, that have the ability to detect multiple potential biomarkers. In the near future, these approaches may lead to the discovery of new biomarkers that, when used with imaging techniques, could help improve our ability to predict the occurrence of acute vascular events.

Key words: *Biomarkers. Atherothrombosis. Proteomics.*

Full English text available from: www.revespcardiol.org

INTRODUCCIÓN

Las enfermedades cardiovasculares son la primera causa de muerte en el mundo occidental[1]. De ellas, la aterosclerosis es el principal motivo de esta enorme morbimortalidad. El proceso patológico que subyace a esta enfermedad es un engrosamiento de la pared arterial debido a la formación de placas ateroscleróticas[2]. Aunque suelen progresar gradualmente, las placas ateroscleróticas se complican frecuentemente con un trombo y ocasionan una obstrucción brusca de la luz vascular. Según su localización, esta oclusión puede dar lugar a síndrome coronario agudo (SCA) o accidente cerebrovascular (ACV), que pueden ocasionar muerte súbita o dejar graves secuelas a quienes lo sufren. Aunque se producen grandes avances en el tratamiento de esta enfermedad, la medicina actual no es capaz de predecir de manera adecuada quiénes tienen riesgo de sufrir estos problemas. Por ello, uno de los mayores retos de la medicina cardiovascular es encontrar la manera de predecir el riesgo de un sujeto de sufrir un evento trombótico agudo.

Fuentes de financiación: Los artículos de los autores citados en el texto se han realizado con ayudas del Ministerio de Ciencia y Tecnología (SAF2007/63648, SAF2007-62533), CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035), Fundación Ramón Areces, FIS 05/0451 y European Network (HEALTH F2-2008-200647).

Correspondencia: Dr. J. Egido.
Servicio de Nefrología e Hipertensión. Laboratorio de Patología Vascular.
Fundación Jiménez Díaz.
Avda. Reyes Católicos, 2. 28040 Madrid. España.
Correo electrónico: Jegido@fjd.es

Durante años, se han utilizado los factores de riesgo cardiovascular para predecir el riesgo de eventos cardiovasculares en la población general, complementándolos con otros datos, como la fracción de eyección. En el caso de pacientes con aterosclerosis sintomática, como en la cardiopatía isquémica, podían añadirse técnicas como la coronariografía para conocer la extensión de la enfermedad. Sin embargo, sigue habiendo una elevada incidencia de eventos isquémicos agudos no esperados, tanto en población con aterosclerosis conocida como en sujetos clasificados como sanos en los que la enfermedad estaba cursando de modo subclínico. En este sentido, uno de los campos de investigación más activos en los últimos años es el uso de la resonancia magnética (RM) y la tomografía computarizada (TC) multicorte. Estas técnicas permiten diagnosticar de modo no invasivo la existencia y la extensión de la aterosclerosis y prometen llegar a una adecuada caracterización del tamaño y la composición de las lesiones. Otro de los grandes campos de investigación en esta área, en el cual nos vamos a centrar, es la búsqueda de biomarcadores diagnósticos y pronósticos que puedan ser identificados en sangre[3]. La pared vascular libera al torrente sanguíneo moléculas que pueden reflejar los procesos patológicos que tienen lugar en ella. Por otra parte, la propia sangre tiene una participación evidente en la formación de trombos. Así, las concentraciones de moléculas que participan en los diferentes procesos patológicos presentes en la aterosclerosis podrían ser biomarcadores en teoría. Sin embargo, no todas estas moléculas son válidas para tal fin, sino que deben reunir ciertas condiciones. Las características de un biomarcador ideal se muestran en la tabla 1. Aunque la mayoría de los biomarcadores estudiados hasta la actualidad se han basado en la posibilidad de que sean útiles desde el punto de vista diagnóstico/pronóstico, conviene recordar que lo ideal sería además que constituyeran una diana terapéutica. Por último algunos, aunque no tengan valor diagnóstico y terapéutico, nos pueden proporcionar información sobre la génesis y la formación de la placa ateromatosa (fig. 1).

TABLA 1. **Características de un biomarcador**

Específico	Para una enfermedad en particular
Sensible	Fácilmente cuantificable
Predictivo	Relevante para la progresión de la enfermedad y/o el tratamiento
Sólido	Rápido, simple y con análisis económicos
Estable	Iguales concentraciones a cualquier hora del día
No invasivo	Fácil obtención de muestras (sangre, orina, etc.)
Relevancia preclínica y clínica	Válido en modelos animales/celulares y humanos

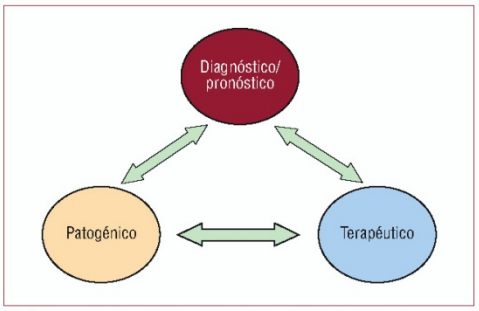


Fig. 1. Tipos de biomarcadores de la enfermedad cardiovascular.

BIOMARCADORES CARDIOVASCULARES

En este apartado se resumen los biomarcadores más estudiados en cuanto a los distintos mecanismos implicados en el desarrollo y la rotura de la placa aterosclerótica, como la disfunción endotelial, la inflamación, el estrés oxidativo, la proteolisis y la trombosis (fig. 2).

Disfunción endotelial

Entre las causas de disfunción endotelial, se encuentran los factores de riesgo cardiovascular y los factores hemodinámicos, pues se sabe que el endotelio se daña en los lugares donde hay más turbulencia de la sangre. En particular destaca el papel de los lípidos, ya que el aumento en su concentración plasmática puede llevar a su acumulación en el espacio subendotelial donde, tras sufrir diversas modificaciones, estimulan la expresión de moléculas de adhesión y se inicia el proceso inflamatorio.

Moleculas de adhesión

Las moléculas de adhesión con clave en el reclutamiento celular hacia el interior de la pared vascular. Dado que sus formas solubles pueden aparecer en el plasma, diversos trabajos han relacionado sus concentraciones con el riesgo de eventos cardiovasculares.

En poblaciones sanas, la molécula de adhesión ICAM-1 *(intercellular adhesion molecule-1)* muestra mayores concentraciones en sujetos sanos que van a tener un infarto agudo de miocardio (IAM), mientras que con la VCAM-1 *(vascular cell adhesion molecule-1)* no se obtuvieron datos similares[4,5]. En el estudio ARIC (Atherosclerosis in Risk Communities), las concentraciones de ICAM-1 predecían eventos coronarios y el desarrollo de aterosclerosis carotídea, y además, había asociación entre ésta y las concentraciones de selectina E soluble[6].

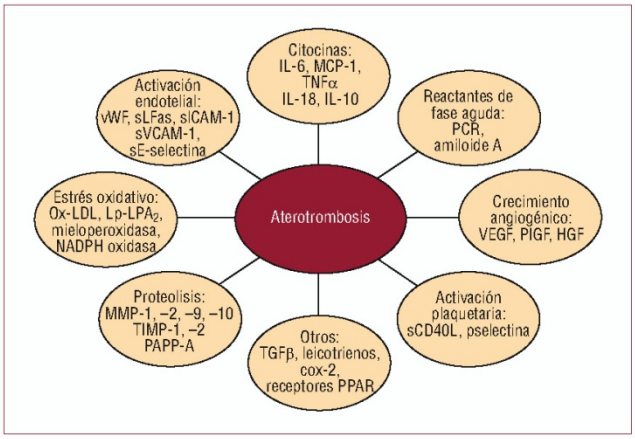


Fig. 2. Marcadores circulantes relacionados con la aterosclerosis. COX-2: ciclooxigenasa 2; HGF: factor de crecimiento de hepatocitos; IL: interleucina; Lp-PLA2: fosfolipasa A2 asociada a lipoproteínas; MCP-1: proteína quimiotáctica de monocitos 1; MMP: metaloproteinasas de matriz; NADPH oxidasa: nicotinamida adenina dinucleótido fosfato oxidasa; ox-LDL: lipoproteínas de baja densidad oxidadas; PAPP-A: proteína plasmática A asociada al embarazo; PCR: proteína C reactiva; PIGF: factor de crecimiento placentario; PPAR: receptores de los activadores de la proliferación de peroxisomas; sE-selectina: selectina E soluble; sLFas: ligando de Fas soluble; sICAM-1: molécula de adhesión intercelular 1 soluble; sVCAM-1: molécula de adhesión a células vasculares-1 soluble; TGFβ: factor de crecimiento transformador beta; TIMP: inhibidor de metaloproteinasas de matriz; TNFα: factor de necrosis tumoral alfa; VEGF: factor de crecimiento del endotelio vascular; vWF: factor de von Willebrand.

En cuanto a la selectina P, el Women's Health Study mostró que era predictora de eventos cardiovasculares[7].

En poblaciones con enfermedad coronaria, el estudio Atherogene mostró que las concentraciones de selectina E, ICAM-1 y VCAM-1 eran mayores en los pacientes que sufrieron eventos cardiovasculares[8]. Mulvihill et al[9] determinaron que la VCAM-1, junto con la proteína C reactiva (PCR), era predictora de eventos cardiovasculares futuros en pacientes con SCA, mientras que ICAM-1 y las selectinas E y P no mostraban ninguna correlación.

Por último, los resultados obtenidos por Malik et al[10] en el British Regional Heart Study son poco alentadores en cuanto al valor pronóstico de las moléculas de adhesión. De los 5.661 varones del estudio, se analizaron muestras de 643 que desarrollaron enfermedad coronaria y 1.278 que permanecieron estables. Basalmente había evidencia de enfermedad coronaria en el 36% de los que tuvieron eventos y el 20% de los que permanecieron estables. Las concentraciones de ICAM-1, VCAM-1 y selectinas E y P no añadieron valor pronóstico al que proporcionaban los factores de riesgo clásicos.

Los datos obtenidos hasta la actualidad acerca del efecto del tratamiento con fármacos hipolipemiantes sobre la concentración plasmática de diferentes moléculas de adhesión son variados. Así, el tratamiento con fluvastatina (80 mg/día) disminuyó las concentraciones plasmáticas de ICAM-1 y selectina P en 26 pacientes hiperlipémicos[11]. Sin embargo, otros estudios no han confirmado estos resultados. Jilma et al[12] analizaron las concentraciones circulantes de ICAM-1, VCAM-1 y selectina E en 75 pacientes con hipercolesterolemia tratados con tres estatinas diferentes durante 3 meses, y no observaron cambios en las concentraciones plasmáticas de las proteínas analizadas. Cabe destacar que esos estudios se realizaron en poblaciones pequeñas; recientemente se han publicado los resultados del estudio AIM (Atorvastatin on Inflammatory Markers)[13], en el que se analizaron las concentraciones plasmáticas de ICAM-1 en 1.078 sujetos con alto riesgo cardiovascular. En ese estudio se observó que el tratamiento durante 3 meses con todas las dosis disponibles de atorvastatina (10-80 mg/día) disminuyó las concentraciones de ICAM-1.

Inflamación

Quimiocinas

Una vez que los leucocitos se han adherido a la pared vascular, su entrada al interior está controlada por quimiocinas. Las dos más numerosas son las y las quimiocinas alfa y beta. Las primeras son quimiotácticas para neutrófilos o linfocitos y entre ellas están las interleucinas (IL). Las quimiocinas beta atraen monocitos y linfocitos, además de basófilos y eosinófilos, aunque no neutrófilos. A esta familia pertenece el MCP-1 *(monocyte chemoattractant protein-1)*.

Interleucina 6

El valor de la IL-6 como predictor de riesgo fue evaluado en el estudio prospectivo de cohortes ABC[14]. En sujetos sin enfermedad vascular, los valores de IL-6 circulante eran predictivos de enfermedad coronaria, insuficiencia cardiaca e ictus. En la angina inestable, Biasucci et al[15] demostraron que los pacientes que presentaron muerte, IAM o angina refractaria durante la hospitalización tenían al ingreso concentraciones de IL-6 más elevadas que

los sujetos que permanecieron estables. En otro estudio de angina inestable, ya con 263 pacientes, las concentraciones de IL-6, junto con las de PCR, predecían la posibilidad de muerte coronaria durante un seguimiento de 17 meses y eran aditivos al valor que proporcionaban los marcadores de daño miocárdico[16]. En el estudio FRISC II (Fragmin and Fast Revascularisation During Instability in Coronary Artery Disease II trial)[17], 3.269 pacientes con SCA fueron aleatorizados al ingreso a tratamiento invasivo o estrategia conservadora. Los valores de IL-6 eran predictores independientes de mortalidad tras un seguimiento de 12 meses. Además, los pacientes que tenían IL-6 elevada eran los que mostraban beneficio al ser asignados a tratamiento agresivo, por lo que la IL-6 podría servir para guiar el tratamiento a emplear en esta población.

MCP-1

Esta quimiocina es la principal encargada del reclutamiento de monocitos a los tejidos en que hay respuesta inflamatoria activa, como es la lesión aterosclerótica. El valor diagnóstico y pronóstico de MCP-1 soluble se ha puesto de manifiesto en diferentes estudios. Las concentraciones plasmáticas de MCP-1 se han asociado con diferentes factores de riesgo cardiovascular, así como con un mayor riesgo de sufrir un evento cardiovascular en el futuro[18,19]. Así en el estudio OPUS-TIMI 16 se analizó su capacidad pronóstica en 2.270 pacientes con SCA sin elevación del segmento ST (SCASEST), y se observó que las concentraciones de MCP-1 predecían el riesgo de muerte o IAM a 10 meses.

Nuestro grupo ha demostrado que el tratamiento con atorvastatina sola o en combinación con amlodipino reduce las concentraciones de MCP-1 en pacientes con aterosclerosis carotídea[20,21]. Además, recientemente hemos publicado los resultados del estudio AIM, en los que se observaba que todas las dosis disponibles de atorvastatina son capaces de disminuir las concentraciones plasmáticas de MCP-1 tras 3 meses de tratamiento en sujetos con alto riesgo cardiovascular[13].

PCR

Sin duda alguna, es el marcador inflamatorio más conocido[22,23]. En pacientes con angina inestable, las concentraciones de PCR fueron predictoras de inestabilidad cardiaca recurrente[24]. De forma similar, la PCR parece ser útil en el manejo diagnóstico y pronóstico de la enfermedad arterial periférica[25]. En pacientes con enfermedad coronaria, la PCR se ha asociado con el riesgo de recurrencia de eventos cardiovasculares[26,27]. Por otro lado, en algunos estudios en pacientes con IAM, las concentraciones de PCR se correlacionan con el tamaño y la extensión de la necrosis, así como con el pronóstico[28]. En prevención primaria, diversos estudios han demostrado que las concentraciones basales de PCR son capaces de predecir eventos vasculares[29-31].

Independientemente de la capacidad de la PCR para predecir el riesgo en prevención primaria y secundaria, el interés sobre la PCR ha aumentado debido a que las estatinas son capaces de disminuir sus concentraciones de forma independiente de su efecto hipolipemiante[32]. En este sentido, en el estudio PROVE IT-TIMI22 (Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22)[33], las concentraciones iniciales de PCR obtenidas tras el tratamiento con estatinas tuvieron igual importancia que las de lipoproteínas de baja densidad (LDL) para predecir eventos cardiovasculares. Cabe destacar los resultados del estudio JUPITER, en el cual se analizó el efecto del tratamiento con rosuvastatina (20 mg/día) en 17.802 sujetos aparentemente sanos que presentaban concentraciones de cLDL < 130 mg/dl y PCR sérica > 2 mg/l, con un seguimiento medio de 1,9 años[34]. En ese estudio, la rosuvastatina disminuyó significativamente la incidencia de eventos cardiovasculares. Sin embargo, es importante notar que el tratamiento con rosuvastatina disminuyó las concentraciones de cLDL por debajo de 55 mg/dl y, por lo tanto, el efecto observado tras el tratamiento podría deberse a la disminución tan drástica observada en la concentración de LDL. En todo caso, hay un gran debate sobre su potencial uso en la práctica clínica[35,36].

Estrés oxidativo

LP-PLA2

La fosfolipasa A2 asociada a lipoproteínas (LP-PLA2) es una lipasa independiente del calcio secretada por leucocitos y está asociada con las LDL circulantes y los macrófagos en la placa ateromatosa. Junto con la PCR, es el predictor de riesgo cardiovascular más sólidamente estudiado. Se han publicado más de 25 estudios epidemiológicos prospectivos sobre la LP-PLA2 tanto en prevención primaria como en secundaria. Estos estudios clínicos han demostrado generalmente correlaciones sólidas entre concentraciones de LP-PLA2 circulante y el aumento del riesgo de eventos cardiovasculares, incluso después del ajuste multivariable para los factores de riesgo tradicionales. Además la LP-PLA2 es un factor de riesgo independiente y complementario de la PCR[37-39]. Esos estudios han respaldado las recomendaciones de la American Heart Association que indican que la LP-PLA2 po-

dría ser utilizada en la práctica clínica para afinar la predicción de riesgo en sujetos con riesgo cardiovascular intermedio.

La LP-PLA2 ha suscitado también mucho interés como diana terapéutica en la enfermedad coronaria. La LP-PLA2 se localiza en altas concentraciones en el núcleo lipídico de las placas inflamatorias. La LP-PLA2 es secretada por las células inflamatorias en la lesión o la transportan las partículas de LDL. La LP-PLA2 actúa sobre la fosfatidilcolina oxidada (localizada en la parte externa de las LDL oxidadas) para generar lisofosfatidilcolina y ácidos grasos oxidados. Estos dos productos lípidos bioactivos inducen la expansión del núcleo lipídico y el adelgazamiento de la capa fibrosa. La inhibición selectiva de la LP-PLA2, mediante el tratamiento con darapladib, inhibe la progresión de las lesiones ateroscleróticas coronarias avanzadas en un modelo experimental[40]. En un estudio realizado en 330 pacientes con enfermedad coronaria documentada angiográficamente, la administración de darapladib durante 12 meses previno la expansión del núcleo necrótico, un determinante clave de la vulnerabilidad de la placa[41]. Aunque son precisos estudios adicionales, estos datos indican que la inhibición de la LP-PLA2 puede ser un nuevo abordaje terapéutico.

Proteolisis

El desequilibrio entre la síntesis y la degradación de la matriz extracelular es un fenómeno clave en el debilitamiento y la rotura de las placas ateroscleróticas avanzadas. Mientras que la muerte por apoptosis de las células de músculo liso vascular (CMLV) parece ser el principal mecanismo involucrado en la disminución de la síntesis de componentes de la matriz, el incremento de su degradación se ha asociado al aumento en las concentraciones y la actividad de diversas enzimas proteolíticas. De estas enzimas, las metaloproteinasas (MMP) han sido las más estudiadas.

Metaloproteinasas

La mayoría de los factores de riesgo de enfermedad aterosclerótica se han asociado a un aumento en las concentraciones de diversas MMP circulantes, entre otras, la hipertensión[42] y la diabetes mellitus[43]. Asimismo, las concentraciones de MMP-9 y del inhibidor TIMP-1 están significativamente aumentadas en pacientes con aterosclerosis carotídea[44,45] y en pacientes con enfermedad coronaria[46]. También se ha detectado un aumento en las concentraciones de ambos marcadores en pacientes que han sufrido un SCA[47]. En relación con el posible valor pronóstico de las MMP, en prevención primaria se ha observado que el aumento en la MMP-9 circulante es capaz de predecir eventos cardiovasculares en sujetos sanos[48]. Asimismo, diversos estudios en prevención secundaria han relacionado las concentraciones de MMP-9 elevadas en pacientes con diversas afecciones cardiovasculares con una mayor mortalidad cardiovascular[49,50].

Entre las diferentes estrategias terapéuticas empleadas en la aterosclerosis que son capaces de modular las concentraciones de MMP, cabe destacar dos: los trombolíticos y las estatinas. Mientras que los trombolíticos pueden estimular la expresión de MMP y la degradación del colágeno[51], las estatinas disminuyen las concentraciones de MMP-9[52].

Trombosis

La presencia de estos procesos inmuno-inflamatorio-proteolíticos en la placa aterosclerótica conduce a la desestabilización, la rotura y la consiguiente formación de trombo, que es la base de las consecuencias clínicas de la aterosclerosis más severas. En el 70% de los enfermos que presentan un SCA ocurre este proceso de rotura de placa. Normalmente se trata de una placa que no estenosa mucho el vaso, contiene grasa y, al producirse una fisura en ella, pone en contacto el núcleo lipídico, rico en factor tisular, con el torrente circulatorio, lo que da lugar a la formación de un trombo que impida el flujo sanguíneo.

CD40/CD40L

Dada la implicación del sistema CD40/CD40L en la aterotrombosis[53,54], también se ha intentado analizar si la determinación de sus valores plasmáticos podría proporcionar información pronóstica. Un aumento de CD40L soluble predice un mayor riesgo de eventos cardiovasculares en mujeres sanas[55]. Sin embargo, la mayor parte de la población que tuvo eventos tenía concentraciones similares a las de quienes permanecieron estables, y la diferencia se debía a un pequeño subgrupo que sí tuvo valores de CD40L claramente más elevados. Por lo tanto, es posible que en mujeres sanas el CD40L soluble pueda distinguir un grupo con especial riesgo de eventos vasculares, pero no a la mayoría.

En pacientes con SCA se ha visto que las plaquetas muestran un incremento de la expresión de CD40L[56]. En el estudio CAPTURE[57], en el que se evaluaba el uso de abciximab frente a placebo, se analizaron las concentraciones de CD40L en pacientes con SCASEST que iban a ser sometidos a angioplastia. En primer lugar, se observó que los pacientes del grupo placebo con concentraciones de CD40L elevadas tenían más probabilidad de muerte

o infarto no fatal durante los 6 meses siguientes. En segundo lugar, el valor predictivo de CD40L era independiente de las concentraciones de troponina T, ya que —incluso en los que se elevaba este marcador— los valores de CD40L seguían teniendo valor pronóstico. Por último, el abciximab reducía el riesgo de eventos en los sujetos con concentraciones de CD40L soluble elevadas hasta igualarlos con el del grupo que tenía valores bajos, mientras que éstos no se beneficiaban del uso de este bloqueador de los receptores IIb/IIIa. Estos hallazgos podrían estar relacionados con que el CD40L soluble confiere una mayor estabilidad al trombo cuando se une a los receptores plaquetarios IIb/IIIa[58], por lo que los pacientes con concentraciones mayores se beneficiarían especialmente de los bloqueadores IIb/IIIa. Datos del estudio OPUS-TIMI16 (Orbofiban in Patients with Unstable coronary Syndromes-Thrombolysis In Myocardial Infarction 16), también en SCASEST, confirman el valor predictivo del CD40L independientemente de las concentraciones de PCR y troponina I[59].

Diferentes trabajos han analizado el efecto del tratamiento hipolipemiante con estatinas en las concentraciones plasmáticas de CD40L. Así, se ha demostrado que el tratamiento con atorvastatina durante 8 semanas disminuye la expresión de CD40L en plaquetas procedentes de sujetos hiperlipémicos[60]. Además, en el estudio ASAP (Atorvastatin versus Simvastatin on Atherosclerotic Progression Study), el tratamiento con atorvastatina (80 mg/día) o simvastatina (40 mg/día) disminuyó las concentraciones plasmáticas de CD40L independientemente de la reducción que se observó en las de colesterol[61]. En el estudio MIRACL (Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering), que reclutó a 2.352 pacientes que habían sufrido un SCA, el tratamiento con atorvastatina (80 mg/día) durante 16 semanas disminuyó las concentraciones de CD40L y redujo el riesgo de padecer un nuevo evento vascular en sujetos que se encontraban en el percentil superior (> 90%)[62]. Finalmente, en el estudio AIM, el tratamiento con todas las dosis disponibles de atorvastatina produjo una disminución de las concentraciones de CD40L circulante en los sujetos con alto riesgo cardiovascular que se encontraban en el cuartil superior[63]. Todos estos estudios indican que el tratamiento hipolipemiante con estatinas es capaz de disminuir las concentraciones de CD40L, probablemente de manera independiente de su acción hipolipemiante.

NUEVOS BIOMARCADORES POTENCIALES

En la búsqueda de nuevos biomarcadores hay dos aproximaciones posibles. La primera es una aproximación clásica basada en la selección de proteínas implicadas en la fisiopatología de la aterotrombosis, como en los ejemplos revisados en la primera parte de esta revisión. En la segunda, mediante el uso de técnicas de alto rendimiento como la proteómica, no se necesita un conocimiento previo de las proteínas ni de la función que pueden desempeñar en la enfermedad. Con esta aproximación podemos comparar fluidos o tejidos de un paciente con los de un sujeto sano y, a modo de rastreo, ver qué proteínas se expresan de modo diferente en una y otra muestra. Se generan así listados de proteínas potencialmente involucradas en esta enfermedad, entre las cuales debemos seleccionar aquellas cuyas función y/o propiedades indiquen que tienen más probabilidades de ser buenos biomarcadores.

Aproximación clásica

Un ejemplo de esta aproximación es el estudio del sistema Fas/ligando de Fas, perteneciente a la superfamilia del receptor del factor de necrosis tumoral. Tanto Fas como ligando de Fas poseen formas solubles y, mientras que Fas soluble (sFas) se genera por *splicing* alternativo de un único gen[64,65], el ligando de Fas soluble (sLFas) se genera mediante la acción de una MMP[66].

Las concentraciones de sLFas están incrementadas en pacientes con insuficiencia cardiaca, IAM o angina inestable[67-69], situaciones agudas en las que las células inflamatorias están muy activadas y podrían aumentar la secreción de esta proteína. Por el contrario, en situaciones crónicas, se ha observado que los pacientes con hiperlipemia familiar combinada o con aterosclerosis carotídea presentan un marcado descenso de las concentraciones de sLFas circulante[70]. La unión de LFas a su receptor produce la activación de la muerte celular programada o apoptosis de la célula que expresa el receptor. Se ha propuesto que la expresión de LFas en algunos tejidos contribuye a un estado de privilegio inmunitario que previene el infiltrado de células inflamatorias, ya que estas células expresan su receptor y, por lo tanto, sufrirían apoptosis cuando entran en contacto con el tejido. En este sentido, se ha demostrado que LFas se produce en las células endoteliales en circunstancias normales y su expresión puede regular negativamente la extravasación celular[71]. Estímulos proinflamatorios, como el factor de necrosis tumoral alfa (TNFα), son capaces de disminuir la expresión de LFas en células endoteliales y facilitar la entrada de células inflamatorias en los estadios tempranos del desarrollo de la lesión aterosclerótica. Nuestra hipótesis es que la disfunción endotelial que tiene lugar en estos pacientes podría ser el motivo de estos hallazgos, probable-

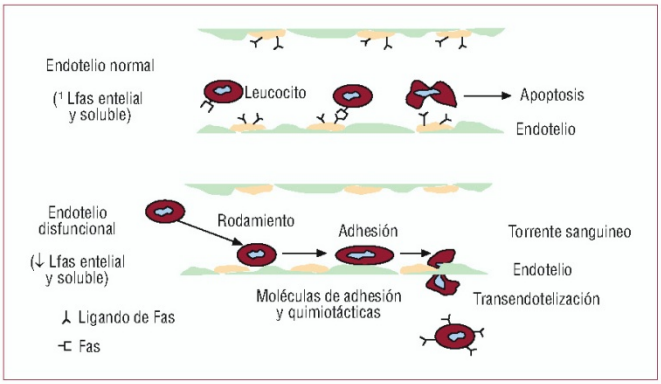


Fig. 3. El ligando de Fas (LFas), un potencial biomarcador de disfunción endotelial. El LFas lo producen y liberan las células endoteliales en circunstancias normales y su expresión puede regular negativamente la extravasación celular mediante la inducción de la apoptosis de leucocitos. Cuando el endotelio es disfuncional, distintos estímulos proinflamatorios son capaces de disminuir la expresión y la liberación de LFas por células endoteliales, lo que facilita la entrada de células inflamatorias en los estadios tempranos del desarrollo de la lesión aterosclerótica.

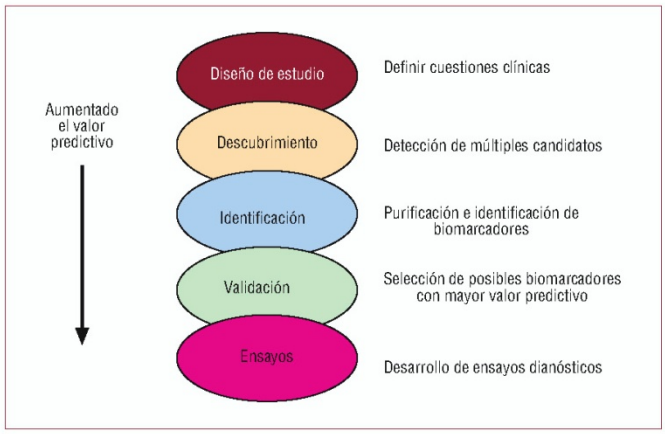


Fig. 4. Cómo identificar un biomarcador.

mente debido a una menor síntesis endotelial y/o una menor liberación a la sangre (fig. 3). Para ello, analizamos las concentraciones de sLFas en 110 pacientes con enfermedad coronaria a los que se determinaba la respuesta vasodilatadora a la hiperemia reactiva como un marcador de función endotelial. Observamos que hay relación lineal entre concentraciones de sLFas e hiperemia reactiva, pero no con el flujo en respuesta a la nitroglicerina (respuesta independiente de endotelio), lo que indica que sLFas puede ser un marcador de función endotelial en pacientes con enfermedad coronaria[72].

Por otro lado, las concentraciones de sFas/sLFas circulantes fueron evaluadas en la población del estudio AIM, el cual reclutó a 1.087 sujetos con alto riesgo cardiovascular. En ese estudio se observó una disminución en las concentraciones de sLFas y un aumento en las de sFas en pacientes con alto riesgo cardiovascular, lo que indicaría que ambas proteínas pueden ser marcadores tempranos de daño vascular. Desafortunadamente, el estudio no se diseñó para evaluar eventos cardiovasculares debido al corto tiempo de seguimiento, por lo que el valor predictivo de sFas y sLFas debe ser puesto a prueba en estudios futuros[73].

La naturaleza compleja del proceso aterotrombótico exige el desarrollo de nuevas tecnologías que permitan descubrir nuevos mediadores involucrados en dicha enfermedad. Aunque no se revisan aquí por limitaciones de espacio, la genómica y la proteómica pueden ser herramientas clave para la identificación de genes y proteínas que confieran una mayor predisposición a los eventos cardiovasculares. Dentro de esta aproximación, es importante definir cuestiones clínicas concretas para la obtención de biomarcadores específicos de la enfermedad en cuestión (fig. 4).

Aproximación proteómica a la búsqueda de nuevos biomarcadores

A continuación se muestran dos ejemplos de nuevos biomarcadores potenciales obtenidos mediante la aplicación de técnicas proteómicas.

Heat shock proteins (HSP)

Las HSP son una familia de proteínas que están presentes en la mayoría de las células. En el interior celular, las HSP actúan como chaperonas facilitando el ensamblaje correcto de las proteínas, así como la translocación de oligómeros, pero también favoreciendo la eliminación de las proteínas que están dañadas de forma irreversible. Por otro lado, pueden ser secretadas y detectadas en plasma. En distintas enfermedades cardiovasculares, se ha observado que su expresión puede modularse tanto en la lesión como en el plasma[74,75]. Varios estudios han analizado las cantidades circulantes de distintas HSP. Así, la HSP60 puede ser un marcador de aterosclerosis[76,77]. Entre las hipótesis del incremento de la HSP60 circulante en aterosclerosis, se ha propuesto su potencial participación en la inmunogenicidad de ciertas bacterias[78] o en el estrés[79]. Las concentraciones de HSP60 y HSP70 están incrementadas en pacientes que han sufrido un SCA, posiblemente en relación con la necrosis del miocardio[80,81]. Por el contrario, las de HSP70 se encuentran disminuidas en pacientes con aterosclerosis[44]. Así, un estudio previo analizó el posible valor pronóstico de la HSP70 circulante en pacientes hipertensos, y observó una correlación inversa entre concentraciones de HSP70 y el grosor íntima-media carotídeo[82]. Esos autores propusieron que las concentraciones de HSP70 elevadas podrían reflejar un estado antiaterogénico en los vasos sanguíneos. Posteriormente, se ha observado que el aumento de las concentraciones de HSP70 se asocia con un menor riesgo de enfermedad coronaria y un menor número de vasos afectados[83].

Mediante una aproximación proteómica, se ha identificado la HSP27 como una proteína que es secretada en gran cantidad por arterias sanas y cuya secreción disminuye hasta concentraciones casi indetectables a medida que aumenta la complejidad de la placa aterosclerótica. Posteriormente, se ha realizado un estudio en el que se evaluaron las concentraciones de HSP27 en un grupo de pacientes con aterosclerosis carotídea y en sujetos sanos de iguales edad y sexo. Las concentraciones de HSP27 circulante estaban significativamente disminuidas en el grupo de pacientes respecto al grupo control[84] (fig. 5). Aunque estos datos preliminares deben ser validados en un grupo más amplio de pacientes y en diversos estadios de la enfermedad cardiovascular,

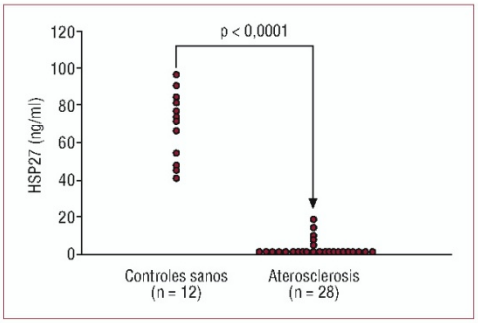


Fig. 5. La HSP27, un potential biomarcador diagnóstico de aterosclerosis. Concentraciones plasmáticas de HSP27 en pacientes con aterosclerosis carotídea (n = 28) y controles sanos (n = 12).

indican que las bajas concentraciones de HPS27 podrían servir como un marcador diagnóstico de enfermedad aterosclerótica avanzada. En cambio, su posible valor pronóstico sólo se ha probado en un estudio en el que se compararon las concentraciones basales de HSP27 de 225 mujeres del Women's Health Study que durante un seguimiento de 6 años sufrieron eventos cardiovasculares y 225 mujeres que no los sufrieron. En ese estudio prospectivo, las concentraciones basales de HSP27 no se asociaron con la aparición de eventos cardiovasculares[85].

TWEAK

El factor de necrosis tumoral inductor débil de apoptosis (*tumor necrosis factor-like weak inducer of apoptosis* [TWEAK, Apo3L, TNFSF12]) pertenece a la superfamilia del TNF[86]. Se considera que esta superfamilia es una fuente de dianas terapéuticas que pueden ser útiles en el manejo de enfermedades complejas. Estas citocinas están implicadas en múltiples respuestas biológicas como la inflamación, la respuesta inmunitaria y la reparación tisular[87]. Los ligandos de esta familia se expresan como proteínas transmembrana tipo II que, en muchos casos, pueden ser procesadas en proteínas secretadas de menor tamaño con actividad biológica[88,89]. Cada ligando se puede unir a uno o más miembros de la superfamilia del TNF, muchos de los cuales son proteínas transmembrana tipo I o III[88,89]. Una vez que el ligando se une al receptor, se activan diferentes cascadas de señalización que promueven múltiples respuestas biológicas.

El TWEAK se expresa ampliamente en diferentes tejidos como páncreas, intestino, corazón, cerebro, pulmón, ovario y músculo esquelético, y en menor medida en hígado y riñón[86-90]. Hay diferentes indicios de que esta proteína puede estar implicada en

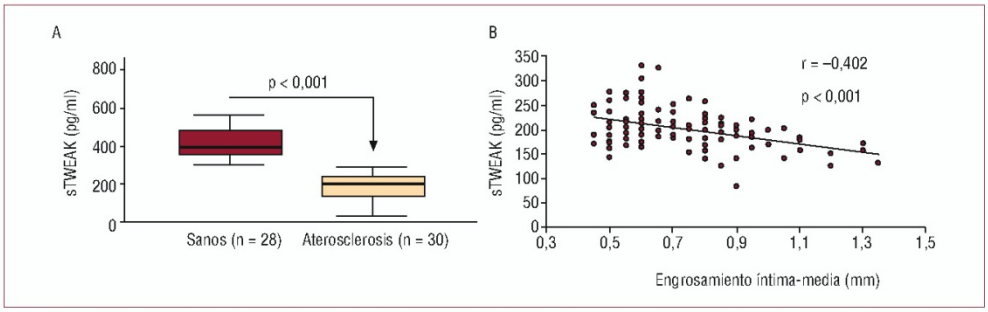


Fig. 6. TWEAK, un biomarcador de aterosclerosis subclínica. Las concentraciones plasmáticas de *tumor necrosis factor-like weak inducer of apoptosis* (TWEAK) están disminuidas en pacientes con aterosclerosis (estenosis carotídea > 70%) (A) y se correlacionan negativamente con el grosor íntima-media carotídeo en sujetos asintomáticos (n = 106) (B).

la patogenia de diferentes enfermedades como aterosclerosis, ictus, artritis reumatoide, daño renal autoinmunitario, daño renal agudo y cáncer[91,92]. Dependiendo del tipo celular analizado, el TWEAK es capaz de estimular proliferación[93], supervivencia[94], migración[95], crecimiento celular[96] y apoptosis[86]. Además, puede promover[91] o inhibir[97] la diferenciación celular. Finalmente, el TWEAK es capaz de inducir la expresión de múltiples proteínas proinflamatorias[98,99].

Usando la tecnología de SELDI-TOF, se ha identificado TWEAK soluble (sTWEAK) como un potencial marcador de aterosclerosis que es secretado por arterias sanas en mayor medida que por placas ateroscleróticas[100]. La cuantificación de sTWEAK en plasma mostró que sus concentraciones están disminuidas en pacientes con aterosclerosis carotídea respecto a los sujetos sanos. Finalmente, el sTWEAK mostró una correlación inversa con el cociente íntima-media en pacientes asintomáticos, lo que indica que esta proteína puede ser un marcador de aterosclerosis subclínica (fig. 6). Estos resultados han sido confirmados posteriormente por Kralisch et al[101], quienes observaron que los sujetos con enfermedad renal crónica o con diabetes mellitus tipo 2, dos cuadros relacionados con alto riesgo de enfermedad cardiovascular, presentaban concentraciones de sTWEAK circulante reducidas.

Además, recientemente se ha demostrado que las concentraciones plasmáticas de sTWEAK predicen mortalidad total y cardiovascular en sujetos sometidos a hemodiálisis[102]. Estos resultados fueron más evidentes cuando las concentraciones plasmáticas de sTWEAK se combinaban con un estado inflamatorio del sujeto (aumento en la concentración plasmática de IL-6). Todos estos resultados parecen indicar que sTWEAK puede ser un muevo biomarcador diagnóstico y pronóstico de aterosclerosis.

CONCLUSIONES

La predicción del riesgo cardiovascular es uno de los grandes desafíos de la medicina moderna. Entre los biomarcadores clásicos revisados, además de los datos de la PCR, los mejores resultados corresponden al ligando de CD40, tanto por su independencia de otras variables como por tratarse de moléculas que participan en la fisiopatología de la aterotrombosis. En este sentido, las concentraciones de CD40L soluble parecen ser más eficaces para valorar el riesgo en situaciones agudas que en prevención primaria. Sin embargo, aún falta estandarización en las determinaciones, y las concentraciones varían claramente de unos estudios a otros. Éste es uno de los factores que hay que solucionar para poder abordar el uso del CD40L como marcador de riesgo en la práctica clínica habitual.

La búsqueda de nuevos biomarcadores mediante técnicas proteómicas permitirá conocer nuevas proteínas que tengan un papel importante en el desarrollo de la enfermedad. Esas proteínas deberán tener poca variabilidad y se debe poder analizarlas con técnicas estándar y con bajo coste económico y de tiempo. Finalmente, el uso de un conjunto de biomarcadores (multimarcador) dará más información del grado de afección del individuo, así como de su pronóstico y su respuesta a un tratamiento. La utilización de estos multimarcadores, en combinación con técnicas de imagen no invasivas, podría dar en el futuro la llave para prevenir las enfermedades cardiovasculares.

BIBLIOGRAFÍA

1. Murray CJ, López AD. Mortality by cause for eight regions of the world: global burden of disease study. Lancet. 1997;349:1269-76.

2. Steinberg D. Atherogenesis in perspective: hypercholesteolemia and inflammation as partner in crime. Nat Med. 2002;11:1211-7.
3. Vivanco F, Martín-Ventura JL, Duran MC, Barderas MG, Blanco-Colio L, Dardé VM, et al. Quest for novel cardiovascular biomarkers by proteomic analysis. J Proteome Res. 2005;4:1181-91.
4. Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet. 1998;351:88-92.
5. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med. 2000;342:836-43.
6. Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation. 1997;96:4219-25.
7. Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. Circulation. 2001;103:491-5.
8. Blankenberg S, Rupprecht HJ, Bickel C, Peetz D, Hafner G, Tiret L, et al. Circulating cell adhesion molecules and death in patients with coronary artery disease. Circulation. 2001;104:1336-42.
9. Mulvihill NT, Foley JB, Murphy RT, Curtin R, Crean PA, Walsh M. Risk stratification in unstable angina and non-Q wave myocardial infarction using soluble cell adhesion molecules. Heart. 2001;85:623-7.
10. Malik I, Danesh J, Whincup P, Bhatia V, Papacosta O, Walker M, et al. Soluble adhesion molecules and prediction of coronary heart disease: a prospective study and meta-analysis. Lancet. 2001;358:971-6.
11. Romano M, Mezzetti A, Marulli C, Ciabattoni G, Febo F, Di Ienno S, et al. Fluvastatin reduces soluble P-selectin and ICAM-1 levels in hypercholesterolemic patients: role of nitric oxide. J Investing Med. 2000;48:183-9.
12. Jilma B, Joukhadar C, Derhaschnig U, Rassoul F, Richter V, Wolzt M, et al. Levels of adhesion molecules do not decrease after 3 months of statin therapy in moderate hypercholesterolaemia. Clin Sci. 2003;104:189-93.
13. Blanco-Colio LM, Martín-Ventura JL, De Teresa E, Farsang C, Gaw A, Gensini G, et al. Elevated ICAM-1 and MCP-1 plasma levels in subjects at high cardiovascular risk are diminished by atorvastatin treatment. Atorvastatin on Inflammatory Markers study: a substudy of Achieve Cholesterol Targets Fast with Atorvastatin Stratified Titration. Am Heart J. 2007;153:881-8.
14. Cesari M, Penninx BW, Newman AB, Kritchevsky SB, Nicklas BJ, Sutton-Tyrrell K, et al. Inflammatory markers and onset of cardiovascular events: results from the Health ABC study. Circulation. 2003;108:2317-22.
15. Biasucci LM, Liuzzo G, Fantuzzi G, Caligiuri G, Rebuzzi AG, Ginnetti F, et al. Increasing levels of interleukin (IL)-1Ra and IL-6 during the first two days of hospitalization in unstable angina are associated with increased risk of in-hospital coronary events. Circulation. 1999;99:2079-84.
16. Koukkunen H, Penttilä K, Kemppainen A, Halinen M, Penttila I, Rantanen T, et al. C reactive protein, fibrinogen, interleukin-6 and tumour necrosis factor-alpha in the prognostic classification of unstable angina pectoris. Ann Med. 2001;33:37-47.
17. Lindmark E, Diderholm E, Wallentin L, Siegbahn A. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. JAMA. 2001;286:2107-13.
18. Deo R, Khera A, McGuire DK, Murphy SA, Meo Neto J de P, Morrow DA, et al. Association among plasma levels of monocyte chemoattractant protein-1, traditional cardiovascular risk factors, and subclinical atherosclerosis. J Am Coll Cardiol. 2004;44:1812-8.
19. De Lemos JA, Morrow DA, Sabatine MS, Murphy SA, Gibson CM, Antman EM, et al. Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndromes. Circulation. 2003;107:690-5.
20. Martín-Ventura JL, Muñoz-García B, Blanco-Colio LM, Martín-Conejero A, Madrigal-Matute J, Vega M, et al. Treatment with amlodipine and atorvastatin has additive effect on blood and plaque inflammation in hypertensive patients with carotid atherosclerosis. Kidney Int Suppl. 2008;S71-4.
21. Martín-Ventura JL, Blanco-Colio LM, Gómez-Hernández A, Muñoz-García B, Vega M, Serrano J, et al. Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. Stroke. 2005;36:1796-800.
22. Tsimikas S, Willerson JT, Ridker PM. C-reactive protein and other emerging blood biomarkers to optimize risk stratification of vulnerable patients. J Am Coll Cardiol. 2006;47 Suppl:C19-31.
23. Casas JP, Shah T, Hingorani AD, Danesh J, Pepys MB. C-reactive protein and coronary heart disease: a critical review. J Intern Med. 2008;264:295-314.
24. Biasucci LM, Liuzzo G, Grillo RL, Caligiuri G, Rebuzzi AG, Buffon A, et al. Elevated levels of C-reactive protein at discharge in patients with unstable angina predict recurrent instability. Circulation. 1999;99:855-60.
25. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Plasma concentration of C-reactive protein and risk of developing peripheral vascular disease. Circulation. 1998;97:425-8.
26. Haverkate F, Thompson SG, Pyke SD, Gallimore JR, Pepys MB. Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. Lancet. 1997;349:462-6.
27. Retterstol L, Eikvar L, Bohn M, Bakken A, Erikssen J, Berg K. C-reactive protein predicts death in patients with previous premature myocardial infarction—a 10-year follow-up study. Atherosclerosis. 2002;160:433-40.
28. Ueda S, Ikeda U, Yamamoto K, Takahashi M, Nishinaga M, Nago N, et al. C-reactive protein as a predictor of cardiac rupture after acute myocardial infarction. Am Heart J. 1996;131:857-60.
29. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation. 2003;107:363-9.
30. Koenig W, Sund M, Fröhlich M, Fischer HG, Löwel H, Döring A, et al. C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. Circulation. 1999;99:237-42.
31. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N Engl J Med. 2002;347:1557-65.
32. Ridker PM, Rifai N, Pfeffer MA, Sacks F, Braunwald E. Long-term effects of pravastatin on plasma concentration of C-reactive protein. The Cholesterol And Recurrent Events (CARE) Investigators. Circulation. 1999;100:230-5.
33. Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, et al. C-reactive protein levels and outcomes after statin therapy. N Engl J Med. 2005;352:20-8.
34. Ridker PM, Danielson E, Fonseca FAH, Genest J, Gotto AM, Kastelein JP, et al. Rosuvastatin to prevent vascular events in men and women with elevated c-reactive protein. N Engl J Med. 2008;359:2195-207.

35. Ridker PM. C-reactive protein and the prediction of cardiovascular events among those at intermediate risk: moving an inflammatory hypothesis toward consensus. J Am Coll Cardiol. 2007;49:2129-38.
36. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med. 2004;350:1387-97.
37. Anderson JL. Lipoprotein-associated phospholipase A (2): An independent predictor of coronary artery disease events in primary and secondary prevention. Am J Cardiol. 2008;101:F23-33.
38. Davidson MH, Corson MA, Alberts MJ, Anderson JL, Gorelick PB, Jones PH, et al. Concensus panel rcommendation for incorporating lipoprotein-associated phospholipase A2 testing into cardiovascular disease risk assessment guidelines. J Am Cardiol. 2008;10:F51-7.
39. Lerman A, McConell JP. Lipoprotein-associated phospholipase A2: A risk marker or a risk factor. Am J Cardiol. 2008;101:F11-22.
40. Wilensky RL, Shi Y, Mohler ER 3rd, Hamamdzic D, Burgert ME, Li J, et al. Inhibition of lipoprotein-associated phospholipase A2 reduces complex coronary atherosclerotic plaque devolpment. Nat Med. 2008;14:1059-66.
41. Serruys PW, García-García HM, Buszman P, Erne P, Verheye S, Aschermann M, et al. Effects of the direct lipoprotein-associated phospholipase A (2) inhibitor darapladib on human coronary atherosclerotic plaque. Circulation. 2008;118:1172-82.
42. Tayebjee MH, Nadar S, Blann AD, Gareth Beevers D, MacFadyen RJ, Lip GY. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in hypertension and their relationship to cardiovascular risk and treatment: A substudy of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT). Am J Hypertens. 2004;17:764-9.
43. Sundstrom J, Evans JC, Benjamin EJ, Levy D, Larson MG, Sawyer DB, et al. Relations of plasma matrix metalloproteinase-9 to clinical cardiovascular risk factors and echocardiographic left ventricular measures. The Framingham Heart Study. Circulation. 2004;109:2850-6.
44. Martín-Ventura JL, Leclercq A, Blanco-Colio LM, Egido J, Rossignol P, Meilhac O, et al. Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. Atherosclerosis. 2007;194:334-41.
45. Beaudeux JL, Giral P, Bruckert E, Bernard M, Foglietti MJ, Chapman MJ. Serum matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1 as potential markers of carotid atherosclerosis in infraclinical hyperlipidemia. Atherosclerosis. 2003;169:139-46.
46. Noji Y, Kajinami K, Kawashiri MA, Todo Y, Horita T, Nohara A, et al. Circulating matrix metalloproteinases and their inhibitors in premature coronary atherosclerosis. Clin Chem Lab Med. 2001;39:380-4.
47. Inokubo Y, Hanada H, Ishizaka H, Fukushi T, Kamada T, Okumura K. Plasma levels of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 are increased in the coronary circulation in patients with acute coronary syndrome. Am Heart J. 2001;141:211-7.
48. Timms PM, Mannan N, Hitman GA, Noonan K, Mills PG, Syndercombe-Court D, et al. Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: mechanisms for inflammatory damage in chronic disorders? Q J Med. 2002;95:787-96.
49. Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, et al. for the AtheroGene Investigators. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation. 2003;107:1579-85.
50. Montaner J, Molina CA, Monasterio J, Abilleira S, Arenillas JF, Ribó M, et al. Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. Circulation. 2003;107:598-603.
51. Host NB, Hansen SS, Jensen LT, Husum D, Nielsen JD. Thrombolytic therapy of acute myocardial infarction alters collagen metabolism. Cardiology. 1994;85:323-33.
52. Koh KK, Son JW, Ahn JY, Jin DK, Kim HS, Choi YM, et al. Comparative effects of diet and statin on NO bioactivity and matrix metalloproteinases in hypercholesterolemic patients with coronary artery disease. Arterioscler Thromb Vasc Biol. 2002;22:e19-23.
53. Mach F, Schönbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, et al. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. Proc Natl Acad Sci U S A. 1997;94:1931-6.
54. Schonbeck U, Libby P. CD40 signaling and plaque instability. Circ Res. 2001;89:1092-103.
55. Schönbeck U, Varo N, Libby P, Buring J, Ridker PM. Soluble CD40L and cardiovascular risk in women. Circulation. 2001;104:2266-8.
56. Garlichs CD, Eskafi S, Raaz D, Schmidt A, Ludwig J, Herrmann M, et al. Patients with acute coronary syndromes express enhanced CD40 ligand/CD154 on platelets. Heart. 2001;86:649-55.
57. Heeschen C, Dimmeler S, Hamm CW, Van den Brand MJ, Boersma E, Zeiher AM, et al. Soluble CD40 ligand in acute coronary syndromes. N Engl J Med. 2003;348:1104-11.
58. Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, et al. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. Nat Med. 2002;8:247-52.
59. Varo N, De Lemos JA, Libby P, Morrow DA, Murphy SA, Nuzzo R, et al. Soluble CD40L: risk prediction after acute coronary syndromes. Circulation. 2003;108:1049-52.
60. Hwang YS, Tsai WC, Lu YH, Lin CC, Chen YF. Effect of atorvastatin on the expression of CD40L and P-selectin in patients with hypercholesterolemia. Am J Cardiol. 2004;94:364-6.
61. Semb AG, Van Wissen S, Ueland T, Smilde T, Waehre T, Tripp MD, et al. Raised serum levels of soluble CD40 ligand in patients with familial hypercholesterolemia: downregulatory effect of statin therapy. J Am Coll Cardiol. 2003;41:275-9.
62. Kinlay S, Schwartz GG, Olsson AG, Rifai N, Sasiela WJ, Szarek M, et al. Effect of atorvastatin on risk of recurrent cardiovascular events after an acute coronary syndrome associated with high soluble CD40 ligand in the myocardial ischemia reduction with aggressive cholesterol lowering (MIRACL) study. Circulation. 2004;110:386-91.
63. Blanco-Colio LM, Martín-Ventura JL, De Teresa E, Farsang C, Gaw A, Gensini G, et al. Atorvastatin decreases elevated soluble CD40L in subjects at high cardiovascular risk. Atorvastatin on inflammatory markers study: a substudy of ACTFAST. Kidney Int. 2008;74:S60-3.
64. Cheng J, Zhou T, Liu C, Shapiro JC, Braeur MJ, Kiefer MC, et al. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science. 1994;263:1759-62.
65. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediates apoptosis. Cell. 1991;66:233-43.
66. Okumara K, Yagita H. Metalloproteinase-mediated release of human Fas ligand. J Exp Med. 1995;182:1777-83.
67. Toyozaki T, Hiroe M, Saito T, Iijima Y, Takano H, Hiroshima K, et al. Levels of soluble Fas in patients with myocarditis, heart failure of unknown origin, and healthy volunteers. Am J Cardiol. 1998;81:798-800.
68. Yamaguchi S, Yamaoka M, Okuyama M, Nitoube J, Fukui A, Shirakabe M, et al. Elevated circulating levels and cardiac secretion of soluble Fas ligand in patients with congestive heart failure. Am J Cardiol. 1999;83:1500-3.

69. Shimizu M, Fukuo K, Nagata S, Suhara T, Okuro M, Fujii K, et al. Increased plasma levels of the soluble form of Fas lignad in patients with acute myocardial infarction and unstable angina pectoris. J Am Coll Cardiol. 2002;39:585-90.
70. Blanco-Colio LM, Martín-Ventura JL, Sol JM, Díaz C, Hernández G, Egido J. Decreased circulating Fas ligand in patients with familial combined hyperlipidemia or carotid atherosclerosis: normalization by atorvastatin. J Am Coll Cardiol. 2004;43:1188-94.
71. Sata M, Walsh K. TNF-a regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation. Nat Med. 1998;4:415-20.
72. Blanco-Colio LM, Martín-Ventura JL, Tuñón J, García-Camarero T, Berrazueta JR, Egido J. Soluble Fas ligand plasma levels are associated with forearm reactive hyperemia in subjects with coronary artery disease: a novel biomarker of endothelial function? Atherosclerosis. 2008;201:407-12.
73. Blanco-Colio LM, Martín-Ventura JL, De Teresa E, Farsang C, Gaw A, Gensini G, et al. Increased soluble Fas plasma levels in subjects at high cardiovascular risk: Atorvastatin on Inflammatory Markers (AIM) study, a substudy of ACTFAST. Arterioscler Thromb Vasc Biol. 2007;27:168-74.
74. Xu Q. Role of heat shock proteins in atherosclerosis. Arterioscler Thromb Vasc Biol. 2002;22:1547-59.
75. Pockley AG. Heat shock proteins, inflammation, and cardiovascular disease. Circulation. 2002;105:1012-7.
76. Pockley AG, Wu R, Lemne C, Kiessling R, De Faire U, Frostegard J. Circulating HSP60 is associated with early cardiovascular disease. Hypertension. 2000;36:303-7.
77. Xu Q, Schett G, Perschinka H, Mayr M, Egger G, Oberhollenzer F, et al. Serum soluble HSP60 is elevated in subjects with atherosclerosis in a general population. Circulation. 2000;102:14-20.
78. Rothenbacher D, Hoffmeister A, Bode G, Miller M, Koenig W, Brenner H. Helicobacter pylori heat shock protein 60 and risk of coronary heart disease: a case control study with focus on markers of systemic inflammation and lipids. Atherosclerosis. 2001;156:193-9.
79. Lewthwaite J, Owen N, Coates A, Henderson B, Steptoe A. Circulating human heat shock protein 60 in the plasma of British civil servants: relationship to physiological and psychosocial stress. Circulation. 2002;106:196-201.
80. Zhang X, He M, Cheng L, Chen Y, Zhou L, Zeng H, et al. Elevated heat shock protein 60 levels are associated with higher risk of coronary heart disease in Chinese. Circulation. 2008;118:2687-93.
81. Dybdahl B, Slørdahl SA, Waage A, Kierulf P, Espevik T, Sundan A. Myocardial ischaemia and the inflammatory response: release of heat shock protein 70 after myocardial infarction. Heart. 2005;91:299-304.
82. Pockley AG, Georgiades A, Thulin T, De Faire U, Frostegard J. Serum HSP70 levels predict the development of atherosclerosis in subjects with established hypertension. Hypertension. 2003;42:235-8.
83. Zhu J, Quyyumi AA, Wu H, Csako G, Rott D, Zalles-Ganley A, et al. Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. Arterioscler Thromb Vasc Biol. 2003;23:1055-9.
84. Martín-Ventura JL, Duran MC, Blanco-Colio LM, Meilhac O, Leclercq A, Michel JB, et al. Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. Circulation. 2004;110:2216-9.
85. Kardys I, Rifai N, Meilhac O, Michel JB, Martín-Ventura JL, Buring JE, et al. Plasma concentration of heat shock protein 27 and risk of cardiovascular disease: a prospective, nested case-control study. Clin Chem. 2008;54:139-46.
86. Chicheportiche Y, Bourdon PR, Xu H, Hsu Y, Scott H, Hession C, et al. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. J Biol Chem. 1997;272:32401-10.
87. Foster D, Parrish-Novak J, Fox B, Xu W. Cytokine-receptor pairing: accelerating discovery of cytokine function. Nature Rev Drug Discov. 2004;3:160-70.
88. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 2001;104:487-501.
89. Bodmer J, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. Trends Biochem Sci. 2002;27:19-26.
90. Marsters SA, Sheridan JP, Pitti RM, Brush J, Goddard A, Ashekenazi A. Identification of a ligand for the death-domain containing receptor Apo3. Curr Biol. 1998;8:525-8.
91. Zheng TS, Burkly LC. No end in site: TWEAK/Fn14 activation and autoimmunity associated end-organ pathologies. J Leukocyte Biology. 2008 [en prensa].
92. Winkles JA. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. Nat Rev Drug Discov. 2008;37:173-8.
93. Donohue PJ, Richards CM, Brown SA, Hanscom HN, Buschman J, Thangada S, et al. TWEAK is an endothelial cell growth and chemotactic factor that also potentiates FGF-2 and VEGF-A mitogenic activity. Arterioscler Thromb Vasc Biol. 2003;23:594-600.
94. Jakubowski A, Browning B, Lukashev M, Sizing I, Thompson JS, Benjamin CD, et al. Dual role for TWEAK in angiogenic regulation. J Cell Sci. 2002;115:267-74.
95. Tran NL, McDonough WS, Donohue PJ, Winkles JA, Berens TJ, Ross KR, et al. The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors. Am J Pathol. 2003;162:1313-21.
96. Wiley SR, Winkles JA. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. Cytokine Growth Factor Rev. 2003;14:241-9.
97. Xu H, Okamoto A, Ichikawa J, Ando T, Tasaka K, Masuyama K, et al. TWEAK/Fn14 interaction stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF. Biochem Biophys Res Commun. 2004;318:422-7.
98. Kim SH, Kang YJ, Kim WJ, Woo DK, Lee Y, Kim DI, et al. TWEAK can induce pro-inflammatory cytokines and matrix metalloproteinase-9 in macrophages. Circ J. 2004;68:396-9.
99. Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S. TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation. J Biol Chem. 2003;278:36005-12.
100. Blanco-Colio LM, Martín-Ventura JL, Muñóz-García B, Meilhac O, Michel JB, Páramo J, et al. Identification of soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) as a possible biomarker of subclinical atherosclerosis. Arterioscler Thromb Vasc Biol. 2007;27:916-22.
101. Kralisch S, Ziegelmeier M, Bachmann A, Seeger J, Lössner U, Blüher M, et al. Serum levels of the atherosclerosis biomarker sTWEAK are decreased in type 2 diabetes and end-stage renal disease. Atherosclerosis. 2008;199:440-4.
102. Carrero JJ, Ortiz A, Qureshi AR, Martín-Ventura JL, Bárány P, Heimbürger O, et al. Additive effects of soluble TWEAK and inflammation on mortality in hemodialysis patients. Clin J Am Soc Nephrol. 2009;4:110-8.

Atherosclerosis 207 (2009) 103–110

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

The CD163-expressing macrophages recognize and internalize TWEAK Potential consequences in atherosclerosis

Juan A. Moreno[a], Begoña Muñoz-García[a], Jose L. Martín-Ventura[a], Julio Madrigal-Matute[a], Josune Orbe[b], Jose A. Páramo[b], Luis Ortega[c], Jesús Egido[a], Luis M. Blanco-Colio[a,*]

[a] Vascular Research Lab, Fundación Jiménez Díaz, Autonoma University, Avda Reyes Catolicos 2, 28040 Madrid, Spain
[b] Atherosclerosis Research Lab, Division of Cardiovascular Sciences, Centre for Applied Medical Research, School of Medicine, Pamplona, Spain
[c] Hospital Clínico San Carlos, Madrid, Spain

ARTICLE INFO

Article history:
Received 12 December 2008
Received in revised form 14 April 2009
Accepted 28 April 2009
Available online 3 May 2009

Keywords:
CD163
TWEAK
Carotid artery
Plasma

ABSTRACT

Background: CD163 is a new potential scavenger receptor of Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) which elicits diverse biologic actions involved in atherosclerosis. We have analyzed the importance of TWEAK–CD163 interaction in atherosclerosis.
Methods: TWEAK and CD163 interaction was studied in cultured human macrophages. Moreover, TWEAK and CD163 expression was analyzed in carotid atherosclerotic plaques (immunohistochemistry) and plasma (ELISA). We have also assessed their potential association with intima/media thickness (IMT) in asymptomatic subjects.
Results: In vitro studies revealed that CD163-expressing macrophages can bind and internalize TWEAK protein exogenously added from supernatants. Accordingly, we observed an inverse correlation between the expression of CD163 and TWEAK ($r = -0.51$; $p = 0.008$) in the shoulder region of atherosclerotic plaques obtained from 25 patients undergoing carotid endarterectomy. The same trend was observed when we analyzed the plasma concentration of both proteins in 90 subjects free from clinical cardiovascular disease ($r = -0.25$; $p = 0.016$) in which carotid ultrasonography was performed to determine IMT. In these subjects, we found a positive correlation between sCD163 and IMT ($r = 0.36$; $p < 0.001$) and between sCD163–sTWEAK ratio and IMT ($r = 0.51$; $p < 0.001$). This association remained significant after adjusting for traditional cardiovascular risk factors and inflammatory markers explaining 39% (sCD163) or 48% (sCD163–sTWEAK ratio) of IMT variance.
Conclusions: Our results suggest that TWEAK–CD163 interaction takes place in vivo, probably decreasing TWEAK plasma concentration. Furthermore, we have observed that CD163–TWEAK plasma ratio is a potential biomarker of clinical and subclinical atherosclerosis.

1. Introduction

CD163 is a 130 kDa member of the scavenger receptor cysteine rich (SRCR) family exclusively expressed on the surface of monocytes/macrophages. It has been identified as the receptor which uptakes haptoglobin–haemoglobin (Hp–Hb) complexes for the removal and metabolism of the potent oxidant haemoglobin [1]. A soluble form of CD163 (sCD163) is a normal constituent in plasma [2] and is generated by proteolytic cleavage (shedding) of CD163 at the cell surface [3]. This receptor is now recognized as an immunomodulator of the atherosclerotic plaque, with pivotal anti-inflammatory and anti-atherogenic properties [4–5].

Recently, CD163 has been identified as a new potential scavenger receptor for tumor necrosis factor-like weak inducer of apoptosis (TWEAK) [6]. TWEAK is a novel member of the TNF superfamily of structurally related cytokines [7]. The first reported TWEAK receptor was fibroblast growth factor-inducible 14 (Fn14) [8–9]. TWEAK is expressed in normal non-atherosclerotic arteries whereas Fn14 expression is almost absent. In contrast, both Fn14 and TWEAK are expressed in atherosclerotic plaques and these proteins colocalize with smooth muscle cells (SMCs) and macrophages [10]. In this context, the interaction between TWEAK and Fn14 has several potential proatherogenic effects in cultured cells which may be important in the pathogenesis of atherosclerosis. TWEAK induces production of proinflammatory cytokines, proliferation and migration of cells present in atherosclerotic plaques and increases the expression of metalloproteinases that degrade the extracellular matrix [11].

Since all these processes play an important role in the pathogenesis of atherosclerosis, we would expect that soluble TWEAK

* Corresponding author.
E-mail address: lblanco@fjd.es (L.M. Blanco-Colio).

0021-9150/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2009.04.033

(sTWEAK) plasma concentration should be elevated in this disease. In contrast, sTWEAK plasma levels are diminished in patients with carotid atherosclerosis [12], or atherosclerosis associated diseases, such as type 2 diabetes or end-stage renal disease [13]. Furthermore, sTWEAK is negatively associated with carotid artery intima/media thickness (IMT) in subjects free from clinical cardiovascular disease [12]. The mechanisms leading to lower sTWEAK plasma levels in subjects with atherosclerosis remain undefined. In this work, we have analyzed the importance of TWEAK–CD163 interaction in human macrophages in vitro and in human atherosclerotic plaque. Furthermore, sCD163 plasma levels were evaluated in 90 asymptomatic subjects in whom IMT and sTWEAK had been previously measured [12].

2. Patients and methods

2.1. In vitro studies

2.1.1. Reagents

RPMI-1640, penicillin and streptomycin were obtained from BioWhittaker. Fetal bovine serum was from Gibco. Recombinant soluble human TWEAK (r-HuTWEAK) was from Alexis. Mouse anti-human Fn14 blocking antibody (ITEM-2) was from eBioscience. r-HuTWEAK was labelled with Cy5 labelling kit (PA35001; Amersham) following the manufacturer's instructions. The remaining reagents were obtained from Sigma unless specified otherwise.

2.1.2. Cell culture

Human monocytic cell line THP-1 (ATCC; CRL-1999) were cultured in RPMI supplemented with 10% decomplemented fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, at 37 °C in 5% CO_2. Cells were differentiated to macrophages using PMA (10^{-7} M) for 48 h. After that, THP-1 macrophages cells were treated for 24 h with dexamethasone (2.5×10^{-7} M) and CD163, TWEAK and Fn14 expression was then assessed by Western blot analysis.

2.1.3. Western blot

Cells from different experimental conditions were collected and pelleted. Western blots were performed as previously described [14]. The blots were incubated with mouse anti-human Fn14 antibody (ITEM-4; eBioscience), goat anti-TWEAK polyclonal antibody (AF1090; R&D Systems) or mouse anti-CD163 monoclonal antibody (EDhu-1; Serotec) and rehybridated with anti-tubulin monoclonal antibody (B-5 to 1–2; Sigma) to confirm equal loading and transfer of proteins. Quantification was expressed as arbitrary densitometric units (ADU).

2.1.4. RNA extraction and real-time polymerase chain reaction

Total RNA was obtained by Trizol method (Life Technologies) and quantified by absorbance at 260 nm in duplicate. Real-time polymerase chain reaction (PCR) was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated *Taq*DNA polymerase (Amplitaq Gold). After an initial hold of 2 min at 50 °C and 10 min at 95 °C, the samples were cycled 40 times at 95 °C for 15 s and 60 °C for 60 s. 18S rRNA served as housekeeping gene and was amplified in parallel with the genes of interest. The expression of target gene was normalized to housekeeping transcripts. Target gene, forward and reverse primers, and probes were designed using Primer Express 1.5 software (Applied Biosystems). All primers, probes, and reagents were obtained from Applied Biosystems. All measurements were performed in duplicate. Values of each sample were obtained as fold to their baseline values.

2.2. Patients with carotid atherosclerosis

Twenty-five consecutive patients (carotid stenosis >70%, 20 men, 5 women; age, 70 ± 7 years; 88% with hypertension, 28% with diabetes, 36% with hyperlipidemia) undergoing carotid endarterectomy at our institutions were included. Informed consent was obtained before enrolment. The study was approved by the local Ethical Committees in accordance with institutional guidelines.

2.3. Subjects free from clinical cardiovascular disease

The population studied consisted of 90 asymptomatic subjects (69 men, age 56.6 ± 12.3 years) in whom global risk assessment was performed in the course of a general health check-up by Internal Medicine Department (University Clinic of Navarra, Spain). The clinical characteristics as well as the prevalence of cardiovascular risk factors had been previously described [12]. In all subjects, absence of history of coronary disease, stroke or peripheral arterial disease was recorded; additional exclusion criteria were the presence of severely impaired renal function, arteritis, connective tissue diseases, alcohol abuse, or use of non-steroidal anti-inflammatory drugs in the 2 weeks before entering the study. Conventional cardiovascular risk factors hypertension, dyslipidemia, diabetes, obesity and smoking were recorded in every patient and were defined as previously described [15,16]. Patients were considered to be hypertensive if they had systolic blood pressure >139 mmHg and/or diastolic pressure >89 mmHg and/or use of anti-hypertensive drugs. Dyslipidemia was diagnosed in the presence of total cholesterol ≥200 mg/dL, LDL cholesterol ≥130 mg/dL, HDL cholesterol <50 mg/dL, triglycerides ≥150 mg/dL and/or use of cholesterol-lowering drugs. Obesity was estimated by the body mass index (BMI ≥ 30 kg/m^2). Diabetes mellitus was defined by fasting glucose levels >126 mg/dL, or by the use of glucose-lowering agents.

The local committee on human research approved the study, which was performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent. In all subjects, carotid ultrasonography was performed to determine IMT, as previously described [15–16]. Subjects were examined by the same two sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination [17]. The between-observer intraclass correlation coefficient was 0.76 ($p < 0.001$) and the between subject repeatability was 0.82 ($p < 0.001$). The corresponding coefficients of variance were 5% and 10%, respectively.

2.4. Enzyme-linked immunosorbent assay

Venous blood samples from different subjects were collected on EDTA. The whole-plasma samples were stored at −80 °C until analysis was performed. Plasma concentrations of sTWEAK and sCD163 were determined in duplicate with commercially available enzyme-linked immunosorbent assay kits (BMS2006INST; Bender MedSystems; S-1015; Bachem; respectively). Plasma samples were assayed in parallel to known standard concentrations of recombinant TWEAK or recombinant CD163. Intra- and inter-assay coefficients of variation were 6.2% and 8.3% (sTWEAK) and 4.2% and 7.3% (sCD163), respectively.

2.5. Immunohistochemistry

Carotid atherosclerotic plaques were fixed in paraformaldehyde for 24 h and stored in ethanol until paraffin-embedded. Immunohistochemistry was performed on 4 μm-thick sections as previously described [18]. Primary antibodies were goat anti-TWEAK polyclonal antibody, mouse anti-CD163 monoclonal

antibody and anti-human macrophages (HAM-56; Dako). For colocalization studies, immunofluorescence was carried out on slides after performing immunohistochemistry for CD163 or macrophages. Negative controls using the corresponding IgG were included for checking non-specific staining.

2.6. Quantification

Computer-assisted morphometric analysis with the Olympus semiautomatic image analysis system Micro Image software (version 1.0 for Windows) was performed by a pathologist (L.O.) as previously described [18–19]. Results are expressed as percentage of positive staining per mm^2.

2.7. Statistical analysis

Statistical analysis was performed with SPSS for Windows software package version 11.0 (SPSS Inc., Chicago, Ill). In vitro experiments were performed at least three times. Results are expressed as mean ± SEM or mean ± SD and were analyzed by ANOVA and Student's t test. Univariate association was performed by Pearson correlation test. Multivariate linear regression analysis was conducted with carotid IMT as dependent variable, including in the model traditional cardiovascular risk factors and inflammatory markers that were significant in the univariate analysis. A two-tailed $p < 0.05$ was considered statistically significant.

3. Results

3.1. CD163 and TWEAK are associated in human carotid atherosclerotic plaques

CD163 and TWEAK expression was analyzed in atherosclerotic plaques from 25 consecutively subjects who underwent carotid endarterectomy. As shown in Fig. 1, both CD163 and TWEAK are present in human carotid atherosclerotic plaques. As expected, CD163 expression was higher in the shoulder (macrophage-rich area) than in the cap region (10.32 ± 1.28 vs 6.97 ± 0.91; respectively; $p < 0.011$). TWEAK expression was not significantly different between shoulder and cap region (8.95 ± 1.49 vs 8.14 ± 1.37; respectively; N.S.). Double immunostaining revealed that TWEAK and CD163 are colocalized by some of the same cells (Fig. 1). When we analyzed the association between CD163 and TWEAK levels in human atherosclerotic plaques we observed an inverse correlation between the expression of both proteins ($r = -0.36$; $p = 0.082$) (Fig. 1B). This inverse association was higher when CD163 and TWEAK expression was only considered in the shoulder region of atherosclerotic plaques ($r = -0.51$; $p = 0.008$) (Fig. 1C).

3.2. CD163-expressing macrophages have less endogenous TWEAK

To assess the relationship between CD163 expression and TWEAK levels, THP-1 cells were treated with PMA (10^{-7} M) for 48 h and then with dexamethasone (DXM) (2.5×10^{-7} M) for 24 h to induce maximal CD163 expression [20]. Cells incubated with PMA or PMA + DXM expressed Fn14 (Fig. 2A). As expected, CD163 was highly expressed in cells in the presence of DXM. Interestingly, TWEAK protein was expressed in macrophages but its expression diminished in the presence of DXM in a time-dependent manner showing an opposite trend to CD163 expression (Fig. 2A). We hypothesize that CD163 could mediate TWEAK uptake and subsequent degradation by macrophages. For this purpose, we used an anti-CD163 blocking antibody to avoid the uptake of endogenous TWEAK. CD163-expressing macrophages that were preincubated with an anti-CD163 blocking antibody (100 ng/mL) showed similar TWEAK levels to non CD163-expressing cells. In order to prevent a possible effect of CD163-neutralizing antibody, we analyzed whether an IgG control induced changes on TWEAK expression; however no differences were observed (data not shown). Furthermore, when CD163-expressing macrophages were incubated in excess of soluble form of r-HuTWEAK (1 ng/mL), endogenous TWEAK levels were re-established (Fig. 2B).

To analyze whether TWEAK diminution is a consequence of TWEAK mRNA downregulation, THP-1 cells were treated with PMA for 48 h and then with dexamethasone for 24 h to induce maximal CD163 expression. At this time (0 h), macrophages exposed to DXM express both CD163 and TWEAK mRNA (Fig. 2C). In addition, CD163 mRNA expression was downregulated in a time-dependent manner (Fig. 2C) although protein expression remained unaltered (Fig. 2A). In contrast, TWEAK mRNA expression remained without changes for up to 24 h, indicating that the diminution in TWEAK protein expression observed in CD163-expressing macrophages was not due to a reduction in mRNA expression.

3.3. CD163-expressing macrophages recognize and internalize sTWEAK in vitro

To analyze whether CD163-expressing macrophages can take up sTWEAK from supernatants, cells were exposed to 1 ng/mL of soluble r-HuTWEAK. As shown in Fig. 3a, quantification of sTWEAK in macrophage supernatants shows a higher consumption by cells incubated with PMA + DXM compared with those with PMA, although non-statistical significance was observed (Fig. 3a). Since Fn14 is present in macrophages independently of CD163 expression, cells were preincubated with an anti-Fn14 blocking antibody for 2 h and then incubated with r-HuTWEAK. In these conditions, sTWEAK levels were decreased only in the supernatants from CD163-expressing macrophages, indicating that CD163 binds and allows uptake of r-HuTWEAK in macrophages (Fig. 3a). Furthermore, when cells were preincubated with both anti-Fn14 and anti-CD163 blocking antibodies, we observed that CD163-expressing macrophages cannot take up r-HuTWEAK from supernatants, indicating a direct interaction between CD163 and r-HuTWEAK. No effects were observed when cells were incubated in the presence of non-specific IgG.

To further examine the cellular interaction between sTWEAK with CD163 and/or Fn14, we labelled r-HuTWEAK with Cy5 fluorescent dye. CD163-expressing macrophages exhibited a binding to TWEAK-Cy5 in both membrane and cytoplasmatic compartments (Fig. 3b-A). To confirm whether CD163 or Fn14 are responsible of TWEAK-Cy5 binding, cells were preincubated with either anti-CD163 or anti-Fn14 blocking antibodies. When cells were exposed to both blocking antibodies, no fluorescence was observed in macrophages (Fig. 3b-B). In addition, when cells were incubated with anti-CD163, TWEAK–Cy5 showed a membrane-staining pattern, indicating its potential interaction with Fn14 (Fig. 3b-C). Moreover, when cells were preincubated with anti-Fn14, TWEAK–Cy5 labelling was present in both, membrane and cytoplasmic compartments, indicating that CD163 can bind and internalize TWEAK protein (Fig. 3b-D). No changes were observed in TWEAK-Cy5 distribution when non-specific IgG were used as a control (not shown).

3.4. CD163–sTWEAK ratio is associated with subclinical atherosclerosis

We have previously reported that sTWEAK plasma levels are diminished and inversely correlated with IMT in asymptomatic subjects [12]. To test whether sCD163 is associated with sTWEAK plasma levels, we have analyzed its plasma levels in 90 asymp-

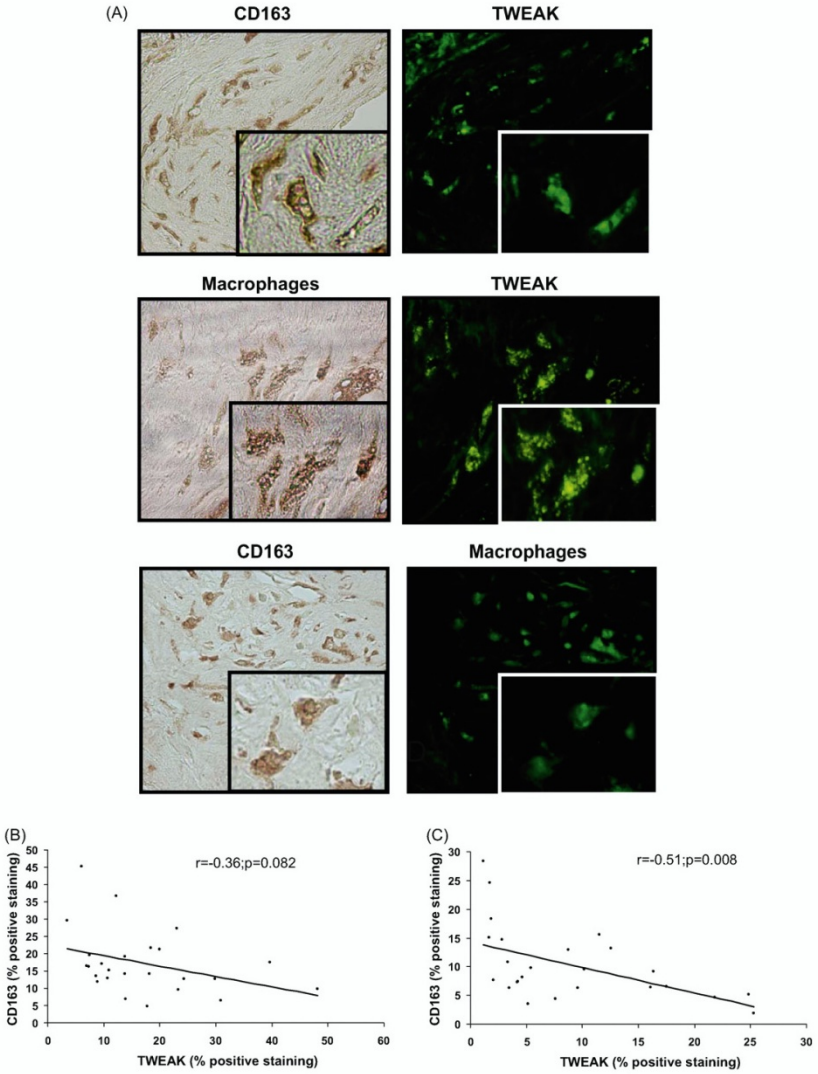


Fig. 1. TWEAK and CD163 expression in human arteries. (A) Colocalization studies showing the expression of CD163, macrophages and TWEAK in sections from human carotid atherosclerotic plaques. Magnification ×200 and ×400. (B) Correlation between TWEAK and CD163 in the whole or (C) in the shoulder region of atherosclerotic plaques.

tomatic subjects in whom IMT and sTWEAK were measured. Characteristics of the studied population are summarized in Table 1, demonstrating variable intensity of conventional atherosclerotic risk factors and anti-atherosclerotic therapy. The percentage of diabetics free from overt cardiovascular disease was 16%, without differences in the sCD163–sTWEAK ratio as compared with non-diabetic patients (13.2 ± 7.8 vs 11.2 ± 6.3). A univariate analysis shows a negative correlation between sCD163 and sTWEAK ($r = -0.25$; $p = 0.016$) (Fig. 4A).

Since sCD163 and sTWEAK show opposite trends, we calculated sCD163–sTWEAK ratio and tested its potential as a marker of subclinical atherosclerosis by evaluating its potential association with IMT. Univariate analysis shows a positive correlation between sCD163 and IMT ($r = 0.36$; $p < 0.001$) and between sCD163–sTWEAK ratio and IMT ($r = 0.51$; $p < 0.001$) (Fig. 4B). As shown in Table I online, no evidence of association between sCD163 or sCD163–sTWEAK ratio and the other clinical parameters was observed. Interestingly, correlation between sCD163–sTWEAK and IMT was superior to that observed between IMT and sCD163 or sTWEAK and other clinical parameters analyzed including C-reactive protein, a marker of systemic inflammation (Table I online).

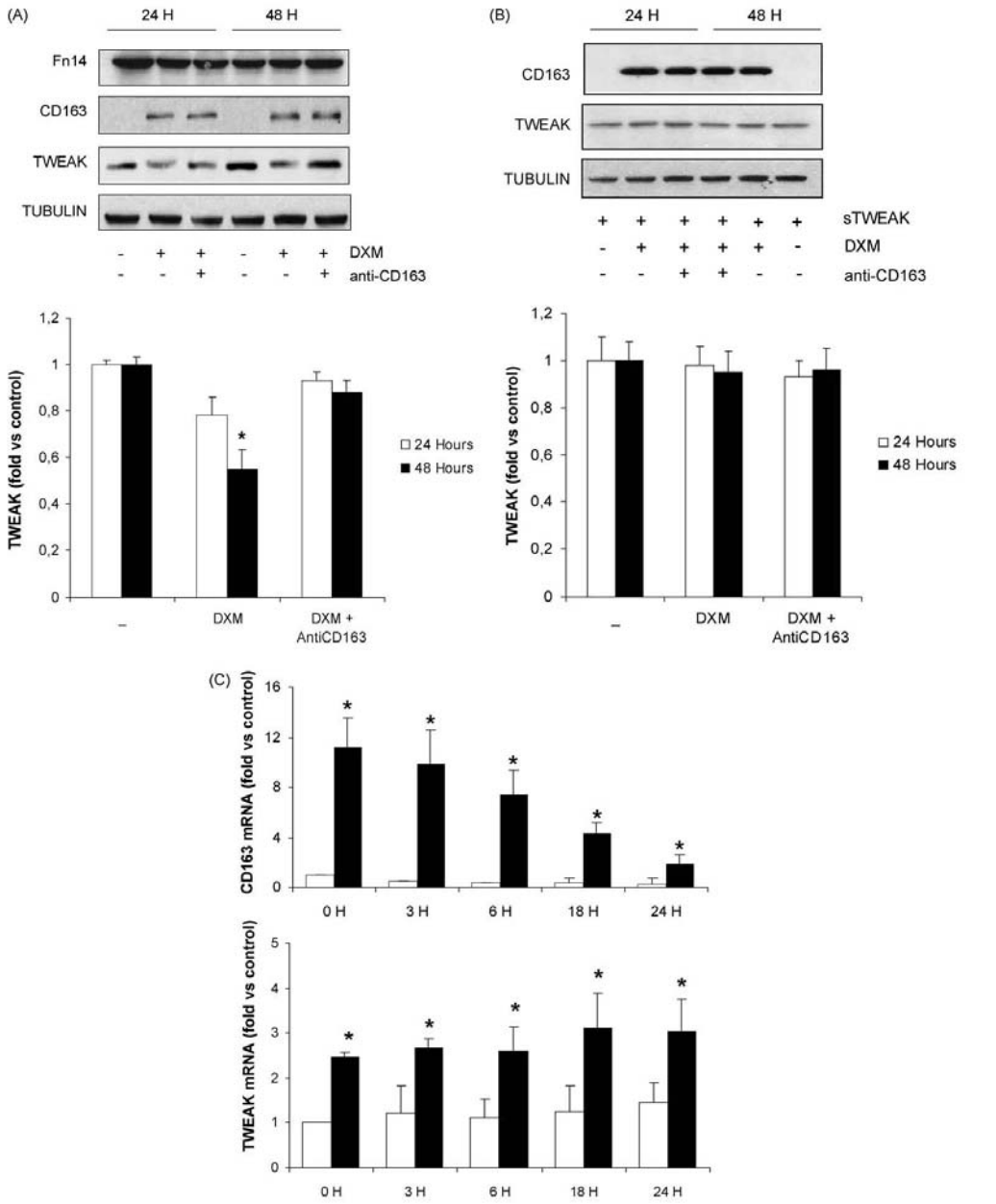


Fig. 2. TWEAK levels in CD163-expressing macrophages. (A) Western blot showing the expression of TWEAK and their receptors Fn14 and CD163 in macrophages incubated with or without DXM and anti-CD163. Results are expressed as mean ± SEM of three independent experiments. (B) Western blot showing TWEAK expression in macrophages in the presence of sTWEAK and incubated with or without DXM and anti-CD163. Results are expressed as mean ± SEM of three independent experiments. (C) Real-time PCR showing the effect of PMA (white) or PMA + DXM (black) on CD163 (upper panel) or TWEAK (lower panel) mRNA expression in macrophages. *$p < 0.05$ vs PMA. Results are expressed as mean ± SEM of four independent experiments.

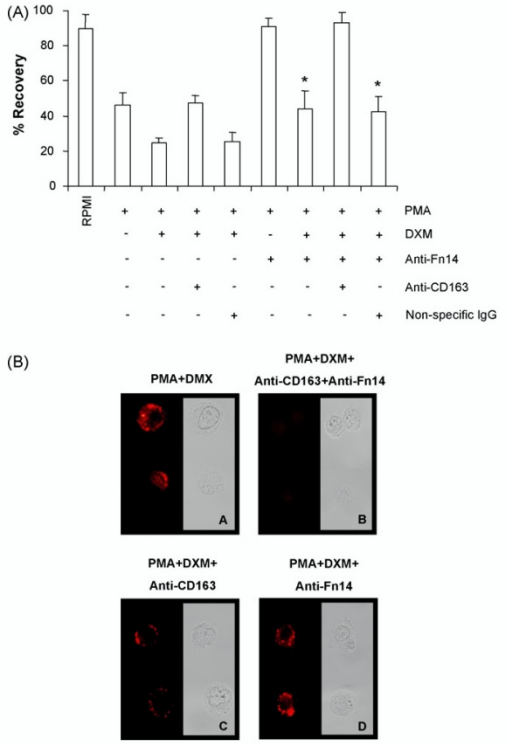


Fig. 3. sTWEAK is sequestered and internalized by CD163-expressing macrophages. (a) Recovery of soluble r-HuTWEAK from supernatants of CD163-expressing macrophages. Soluble r-HuTWEAK (1 ng/mL) was incubated with CD163-expressing macrophages in absence or presence of both anti-CD163 and anti-Fn14 blocking antibodies during 6 h. After that, sTWEAK levels were measured by ELISA in supernatants. (b) In situ detection of TWEAK binding to CD163-expressing macrophages. rTWEAK-Cy5 staining in macrophages incubated PMA and DXM (A) in presence of anti-CD163 (C), anti-Fn14 (D) or both antibodies (B).

Table 1
Baseline clinical characteristics of the studied populations.

Total population ($n = 90$)	
Age (years)	56.6 ± 12.3
Sex (male/female)	69/21
Smokers (yes/no)	27/63
BMI (kg/m^2)	28.1 ± 3.6
SBP (mmHg)	132.6 ± 24.4
DBP (mmHg)	81.6 ± 10.7
Arterial hypertension (yes/no)	49/41
Diabetes mellitus (yes/no)	15/75
Glucose (mg/dL)	108.9 ± 36.4
Total cholesterol (mg/dL)	216.0 ± 39.6
HDL-cholesterol (mg/dL)	49.8 ± 13.6
LDL-cholesterol (mg/dL)	143.3 ± 34.4
Triglycerides (mg/mL)	113.9 ± 58.8
CRP (mg/L)[a]	4.8 ± 0.8
Fibrinogen (mg/mL)	319.8 ± 91.0
vWF (%)	124.2 ± 75.9
sTWEAK (pg/mL)	200.1 ± 43.3
sCD163 (ng/mL)	2145.2 ± 1017.0
sCD163–sTWEAK ratio[a]	11.5 ± 6.6 (0.7)
Mean carotid IMT (mm)	0.74 ± 0.2

BMI indicates body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; IMT, intima-media thickness; SBP, systolic blood pressure; vWF, von Willebrand factor.
[a] Values are expressed as mean ± SD, number of subjects and mean ± SEM.

The association between sCD163 or sCD163–sTWEAK ratio and carotid IMT remained significant after adjusting for traditional risk factors and inflammatory markers, explaining 39% (sCD163) or 48% (sCD163–sTWEAK ratio) of IMT variance (Table II online).

4. Discussion

Our results show that CD163 and TWEAK are expressed in an opposite trend in human carotid atherosclerotic plaques and that CD163-expressing macrophages are able to bind and internalize sTWEAK in vitro. More importantly, sCD163 plasma levels are negatively associated with sTWEAK concentrations and sCD163–sTWEAK ratio is positively correlated with IMT in asymptomatic subjects.

CD163 is a member of the class B scavenger receptors restricted to monocyte/macrophages lineage, which is expressed as a membrane protein and actively shed from the cell surface [21]. The principal characteristic of CD163 is to be a scavenger receptor for Hp–Hb complex. Hp proteins represents the first line of defense against the toxic effects of free Hb. Hp binds to free Hb forming a Hp–Hb complex which may be cleared by CD163 receptor [22–23].

Clearance of the Hp–Hb complex from the atherosclerotic plaque is mediated exclusively by CD163. In addition to its scavenger function, this receptor is now recognized as an immunomodulator of the atherosclerotic plaque, with anti-inflammatory and anti-atherogenic properties [4–5]. In agreement with previous reports in which CD163 expression has been shown in coronary and aortic lesions [20,24], we have observed that CD163 is

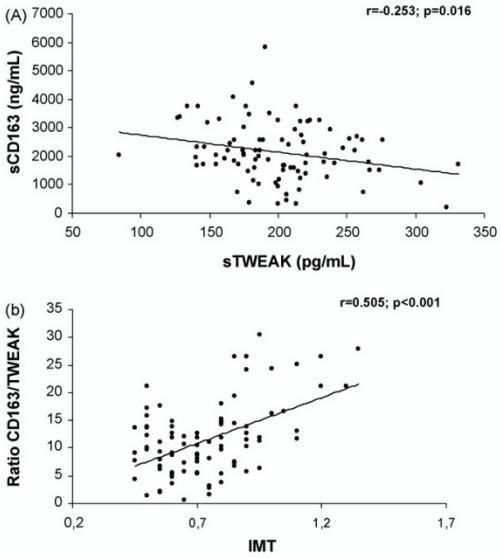


Fig. 4. (A) Correlation between sCD163 and sTWEAK in asymptomatic individuals. sCD163 negatively correlates with sTWEAK concentration ($r = -0.25$; $p = 0.016$). (B) IMT and sCD163–sTWEAK ratio in asymptomatic individuals. The IMT of common carotid arteries correlates with sCD163 concentration ($r = 0.36$; $p < 0.001$) and sCD163–sTWEAK ratio ($r = 0.51$; $p < 0.001$).

expressed in human carotid atherosclerotic plaques, principally in the shoulder region, an area characterized by a high inflammatory content [18]. CD163 has been recently identified as a new receptor for TWEAK [6], a novel member of the TNF superfamily. Through interaction with its receptor Fn14, TWEAK increases the production of different molecules such as proinflammatory cytokines and metalloproteinases, and activates proliferation, migration and angiogenesis [11]. In this context, we have previously reported that TWEAK is expressed by both SMCs and macrophages within atherosclerotic plaques [12]. In the present study, we showed that TWEAK is present in human carotid atherosclerotic plaques colocalizing with CD163. Moreover, we have observed an inverse association between CD163 and TWEAK expression in the shoulder of atherosclerotic plaques, indicating that co-expression or interaction between CD163 and TWEAK can take place in vivo.

It has been demonstrated that TWEAK mediates signal transduction in cells lacking Fn14, suggesting the existence of an alternative TWEAK receptor [25]. In this respect, CD163 from CD14-positive monocytes not expressing Fn14 has been related with TWEAK binding [6]. However, the functionality of CD163 in cells expressing Fn14 has not been yet analyzed. Since CD163 and TWEAK colocalize within atherosclerotic plaques as we previously reported for TWEAK and Fn14 [10], we evaluated the potential significance of CD163–TWEAK interaction in cells-expressing both receptors, CD163 and Fn14. We have observed that Fn14–CD163-expressing macrophages have less amount of endogenous TWEAK protein compared with those without CD163. Furthermore, TWEAK mRNA expression was not affected and remained constant in Fn14–CD163-expressing macrophages, suggesting that TWEAK protein diminution was not related with its mRNA expression. Interestingly, this diminution was reversed either by an anti-CD163 blocking antibody or in competition studies with soluble r-HuTWEAK in the culture medium, indicating that CD163–TWEAK interaction was responsible of endogenous TWEAK reduction.

Moreover, we have shown that CD163-expressing macrophages can sequester and internalize sTWEAK from culture medium. Recovery experiments demonstrated that both Fn14 and CD163 can sequester soluble r-HuTWEAK from the culture medium. When cells were preincubated with anti-Fn14 or anti-CD163 blocking antibody, we observed an increment in soluble r-HuTWEAK recovery from the culture medium. Moreover, when both blocking antibodies were present in the culture medium soluble r-HuTWEAK was not sequestered by macrophages. Consistently with these results, staining of a soluble form of r-HuTWEAK labelled with Cy5 showed that macrophages in the presence of an anti-Fn14 antibody could internalize sTWEAK, probably through interaction with CD163. In contrast, macrophages incubated with an anti-CD163 antibody were capable of binding sTWEAK but could not internalize it, indicating that sTWEAK was bound to Fn14. In addition, macrophages incubated with both anti-CD163 and anti-Fn14 antibodies could not bind sTWEAK, indicating that macrophages could only interact with sTWEAK by either CD163 or Fn14 since both receptors are functional in cells co-expressing them. Furthermore, internalization of sTWEAK by CD163 could be followed by its degradation, as observed in CD163-expressing cells without Fn14 expression [6]. In this context, it is important to note that TWEAK protein structure mimics the Hp–Hb complex [6], indicating that TWEAK could compete with Hp–Hb for CD163 binding. In this condition, the presence of sTWEAK could have deleterious consequences since it could induce an augmentation of free Hp–Hb levels, increasing the pathological effects of this complex in the arterial wall [4]. However, future research efforts regarding TWEAK–CD163 interaction could help to address this hypothesis.

As commented above, CD163 has been shown to be present in a natural soluble form in plasma [2]. Because of a constant shedding of the receptor from the membrane, the plasma concentration of the receptor may reflect its general expression level [2]. The elevation of sCD163 in diseases associated with macrophage activation has been described. Patients with rheumatoid arthritis [26], Gaucher disease [27], hemophagocytosis [28], sepsis [29], and myelomonocytic leukaemia [29] have increased soluble CD163 levels in plasma relative to healthy controls. Furthermore, sCD163 concentrations have been a predictor of coronary artery disease extent independently of conventional risk factors such as age, hyperlipidemia, hypertension and smoking status [30]. Since sTWEAK plasma levels concentrations are diminished in subjects with carotid atherosclerosis, diabetes or chronic kidney disease [12–13] and are negatively associated with IMT, an index of subclinical atherosclerosis [12], we have explored whether CD163–TWEAK interaction could be reflected by sCD163 and sTWEAK plasma concentrations. We have shown that sCD163 plasma levels inversely correlated with sTWEAK concentrations in asymptomatic subjects in whom IMT were measured [12]. These data are in agreement with the observation that subjects with diabetes have increased sCD163 plasma levels [20] and decreased sTWEAK concentrations [13] compared with those without diabetes. Moreover, sCD163 plasma levels were associated with IMT in asymptomatic subjects and this association remained significant after adjustment by traditional cardiovascular risk factors and other inflammatory biomarkers. In addition, when considered sCD163–sTWEAK ratio, an even higher association with IMT was observed. Whether sCD163–sTWEAK ratio could be a potential novel biomarker of atherosclerosis warrants further investigation in prospective studies.

In conclusion, our results shows that TWEAK–CD163 interaction could occur within atherosclerotic plaques and CD163 could be responsible of sTWEAK plasma diminution observed in subjects with cardiovascular disease. Furthermore, sCD163–sTWEAK ratio could be a potential biomarker of atherosclerosis in asymptomatic subjects.

Acknowledgment

This work was supported by grants from Fondo de Investigaciones Sanitarias (CP04/00060), Ministerio de Educación y Ciencia (SAF2007-60896, SAF2007-63648), Comunidad de Madrid (S2006-GEN-0247) and Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (RD06/0014/0035).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.04.033.

References

[1] Kristiansen M, Graversen JH, Jacobsen C, et al. Identification of the haemoglobin scavenger receptor. Nature 2001;409:198–201.
[2] Møller HJ, Peterslund NA, Graversen JH, Moestrup SK. Identification of the hemoglobin scavenger receptor/CD163 as a natural soluble protein in plasma. Blood 2002;99:378–80.
[3] Droste A, Sorg C, Högger P. Shedding of CD163, a novel regulatory mechanism for a member of the scavenger receptor cysteine-rich family. Biochem Biophys Res Commun 1999;256:110–3.
[4] Guetta J, Strauss M, Levy NS, Fahoum L, Levy AP. Haptoglobin genotype modulates the balance of Th1/Th2 cytokines produced by macrophages exposed to free hemoglobin. Atherosclerosis 2007;191:48–53.
[5] Abraham NG, Drummond G. CD163 mediated hemoglobin-heme uptake activates macrophage HO-1, providing an anti-inflammatory function. Circ Res 2006;99:911–4.
[6] Bover LC, Cardo-Vila M, Kuniyasu A, et al. A previously unrecognized protein-protein interaction between TWEAK and CD163: potential biological implications. J Immunol 2007;178:8183–94.

[7] Chicheportiche Y, Bourdon PR, Xu H, et al. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. J Biol Chem 1997;272:32401–10.
[8] Nakayama M, Harada N, Okumura K, Yagita H. Characterization of murine TWEAK and its receptor (Fn14) by monoclonal antibodies. Biochem Biophys Res Commun 2003;306:819–25.
[9] Wiley SR, Winkles JA. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. Cytokine Growth Factor Rev 2003;14:241–9.
[10] Muñoz-García B, Martín-Ventura JL, Martínez E, et al. Fn14 is upregulated in cytokine-stimulated vascular smooth muscle cells and is expressed in human carotid atherosclerotic plaques: modulation by atorvastatin. Stroke 2006;37:2044–53.
[11] Blanco-Colio LM, Martín-Ventura JL, Munoz-Garcia B, et al. TWEAK and Fn14. New players in the pathogenesis of atherosclerosis. Front Biosci 2007;12:3648–55.
[12] Blanco-Colio LM, Martín-Ventura JL, Muñóz-García B, et al. Identification of soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) as a possible biomarker of subclinical atherosclerosis. Arterioscler Thromb Vasc Biol 2007;27:916–22.
[13] Kralisch S, Ziegelmeier M, Bachmann A, et al. Serum levels of the atherosclerosis biomarker sTWEAK are decreased in type 2 diabetes and end-stage renal disease. Atherosclerosis 2008;199:440–4.
[14] Blanco-Colio LM, Munoz-Garcia B, Martin-Ventura JL, et al. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors decrease Fas ligand expression and cytotoxicity in activated human T lymphocytes. Circulation 2003;108:1506–13.
[15] Greenland P, Smith SC, Grundy SM. Improving coronary heart disease assessment in asymptomatic people. Role of traditional risk factors and non-invasive cardiovascular tests. Circulation 2001;104:1863–7.
[16] Beloqui O, Paramo JA, Orbe J, et al. Monocyte cyclooxygenase-2 overactivity: a new marker of subclinical atherosclerosis in asymptomatic subjects with cardiovascular risk factors? Eur Heart J 2005;26:153–8.
[17] Paramo JA, Orbe J, Beloqui O, et al. Prothrombin fragment 1_2 is associated with carotid intima-media thickness in subjects free of clinical cardiovascular disease. Stroke 2004;35:1085–9.
[18] Martin-Ventura JL, Blanco-Colio LM, Munoz-Garcia B, et al. NF-kappaB activation and Fas ligand overexpression in blood and plaques of patients with carotid atherosclerosis: potential implication in plaque instability. Stroke 2004;35:458–63.
[19] Martin-Ventura JL, Blanco-Colio LM, Gomez-Hernandez A, et al. Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. Stroke 2005;36:1796–800.
[20] Levy AP, Purushothaman R, Levy NS, et al. Downregulation of the hemoglobin scavenger receptor in individuals with diabetes and Hp 2-2 genotype. Implications for the response to intraplaque hemorrahge and plaque vulnerability. Circ Res 2007;101:106–10.
[21] Fabriek BO, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor CD163. Immunobiology 2005;210:153–60.
[22] Kristiansen M, Graversen JH, Jacobsen C, et al. Identification of the hemoglobin scavenger receptor. Nature 2001;409:198–201.
[23] Graversen JH, Madsen M, Moestrup SK. CD163: a signal receptor scavenging haptoglobin hemoglobin complexes from plasma. Int J Biochem Cell Biol 2002;234:309–14.
[24] Schaer CA, Schoedon G, Imhof A, Kurrer MO, Schaer DJ. Constitutive endocytosis of CD163 mediates hemoglobin-heme and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. Cir Res 2006;99:943–50.
[25] Polek TC, Talpaz M, Darnay BG, Spivak-Kroizman T. TWEAK mediates signal transduction and differentiation of RAW264.7 cells in the absence of Fn14/TweakR. Evidence for a second TWEAK receptor. J Biol Chem 2003;278:32317–23.
[26] Matsushita N, Kashiwagi M, Wait R, et al. Elevated levels of soluble CD163 in sera and fluids from rheumatoid arthritis patients and inhibition of the shedding of CD163 by TIMP-3. Clin Exp Immunol 2002;130:156–61.
[27] Møller HJ, de Fost M, Aerts H, Hollak C, Moestrup SK. Plasma level of the macrophage-derived soluble CD163 is increased and positively correlates with severity in Gaucher's disease. Eur J Haematol 2004;72:135–9.
[28] Schaer DJ, Schleiffenbaum B, Bächli EB. Soluble hemoglobin–haptoglobin scavenger receptor CD163 as a lineage specific marker in the reactive hemophagocytic syndrome. Eur J Haematol 2005;74:6–10.
[29] Walter RB, Bächli EB, Schaer DJ, Rüegg R, Schoedon G. Expression of the hemoglobin scavenger receptor (CD163/HbSR) as immunophenotypic marker of monocytic lineage in acute myeloid leukemia. Blood 2003;101:3755–6.
[30] Aristoteli LP, Møller HJ, Bailey B, Moestrup SQ, Kritharides L. The monocytic lineage specific soluble CD163 is a plasma marker of coronary atherosclerosis. Atherosclerosis 2006;184:342–7.

Cardiovascular Research (2009) 83, 586–594
doi:10.1093/cvr/cvp141

Increased CD74 expression in human atherosclerotic plaques: contribution to inflammatory responses in vascular cells

José Luis Martín-Ventura[1]*, Julio Madrigal-Matute[1], Begoña Muñoz-Garcia[1], Luis Miguel Blanco-Colio[1], Melany Van Oostrom[1], Guillermo Zalba[2], Ana Fortuño[2], Carmen Gomez-Guerrero[1], Luis Ortega[3], Alberto Ortiz[1], Javier Diez[2,4], and Jesús Egido[1]

[1]*Renal and Vascular Research Lab, Fundación Jimenez Diaz, Autonoma University, Madrid, Spain;* [2]*Division of Cardiovascular Sciences, Center for Applied Medical Research University of Navarra, Pamplona, Spain;* [3]*Anatomy Pathology Services, Hospital Clínico de San Carlos, Universidad Complutense, Madrid, Spain; and* [4]*Department of Cardiology and Cardiovascular Surgery, University Clinic, University of Navarra, Pamplona, Spain*

Received 2 December 2008; revised 13 April 2009; accepted 27 April 2009; online publish-ahead-of-print 7 May 2009

Time for primary review: 13 days

KEYWORDS
Atherosclerosis;
Inflammation;
Biomarkers;
Carotid stenosis

Aims The purpose of this study was to analyse the expression of CD74 in human atherosclerotic plaques and peripheral blood mononuclear cells (PBMC) as well as its potential participation in proinflammatory responses in cultured human vascular smooth muscle cells (VSMC).

Methods and results CD74 expression was analysed in human atherosclerotic plaques (immunohistochemistry), PBMC (real-time PCR), and human aortic VSMC (real-time PCR and western blotting). Nuclear factor-κB (NF-κB) activation was assessed by southwestern histochemistry and electrophoretic mobility shift assay. Monocyte chemoattractant protein-1 (MCP-1) levels were studied by both real-time PCR and enzyme-linked immunosorbent assay. CD74 immunostaining was increased in the inflammatory vs. the fibrous region of atherosclerotic plaques ($n = 70$, 18.2 ± 1.3 vs. $7.8 \pm 0.6\%$ positive staining/mm^2, $P < 0.001$). CD74 colocalized with the transcription factor NF-κB in both VSMC and macrophages. In cultured VSMC, CD74 expression was induced by interferon γ (IFNγ). Incubation with an agonistic anti-CD74 antibody or with IFNγ elicited MCP-1 expression, which was prevented by AKT and γ-secretase inhibitors. Moreover, CD74 small-interfering RNA decreased NF-κB activation and MCP-1 production induced by IFNγ in VSMC. Finally, CD74 mRNA levels in PBMC from patients with carotid stenosis were higher than in healthy subjects ($n = 20$, 3 ± 0.5 vs. 2 ± 0.5 AU, $P < 0.001$). Additionally, a linear trend between CD74 mRNA expression tertiles and intima-media thickness (IMT) was observed in PBMC from asymptomatic subjects ($n = 185$, $P < 0.001$).

Conclusion CD74 levels are increased in plaques and PBMC from patients with carotid stenosis and are associated with IMT in subjects free from clinical cardiovascular diseases. CD74 could be a novel therapeutic target to decrease the inflammatory response in atherosclerosis.

1. Introduction

CD74 (invariant polypeptide of major histocompatibility complex, MHC, HLA-DR gamma) is a non-polymorphic type II integral membrane protein which was thought to function mainly as an MHC class II chaperone, although 2–5% is expressed independent of MHC class II at the cell membrane.[1] In this respect, CD74 has been recently shown to have a role as an accessory-signalling molecule. Stimulation of surface CD74 induces a signalling cascade resulting in AKT and nuclear factor-kB (NF-κB) activation, as well as cell proliferation.[2] Interestingly, NF-kB can be activated by the intracellular domain of CD74, which is liberated from the membrane.[3] This process of intramembrane cleavage followed by nuclear translocation and transcriptional activation is known as regulated-intramembrane proteolysis (RIP).[4] The initial step of ectodomain shedding is carried out by one or more metalloproteases (ADAM or MMPs), yielding a fragment which is substrate for another intramembrane proteases (γ-secretase/presenilins or SPP-type aspartic proteases). In addition, CD74 was found to be a putative receptor for macrophage inhibitor factor (MIF), implicating CD74 in MIF-induced activation of the MAP kinase cascade, cell proliferation, and prostaglandin E_2 production in macrophages.[5] More recently, chemokine receptors (CXCR2 and CXCR4) have been identified as new

* Corresponding author.
E-mail address: jlmartin@fjd.es

functional receptors for MIF.[6] In this regard, inflammatory cell recruitment induced by MIF relied on both CXCR2 and CD74, although independent effects of CD74 were also suggested. Furthermore, *Helicobacter pylori* was shown to interact with CD74, stimulating the production of the chemokine interleukin-8.[7] All these data support a potential role of CD74 in inflammatory diseases.

The early events of atherosclerosis include the recruitment of lipoproteins and inflammatory cells to the vessel wall and the proliferation of vascular cells. In these processes, there are many important mediators, such as interferon gamma (IFNγ) and monocyte chemoattractant protein-1 (MCP-1). Among other proatherogenic properties, IFNγ is able to induce vascular smooth muscle cell (VSMC) activation,[8] and MCP-1 plays a crucial role in monocyte recruitment to the vascular wall.[9] In this respect, the vulnerable region of human atherosclerotic plaques is characterized by an increase in macrophage infiltration, NF-kB activation, and MCP-1 expression.[10,11] Since CD74 has been previously related with monocyte infiltration in experimental atherosclerosis[6] and increased CD74 mRNA levels in circulating blood cells have been recently observed in different pathologies where inflammation plays an important role,[12,13] we firstly performed an observational study to address the presence and localization of CD74 in human atherosclerotic plaques. To test the hypothesis that CD74 could be directly linked to monocyte recruitment in atherothrombosis, further studies in human vascular cells were performed addressing the involvement of CD74 in MCP-1 induction, analysing the potential mechanisms implicated. Finally, on the basis of the importance of inflammation in atherogenesis, recent work has focused on whether circulating markers of inflammation can non-invasively diagnose and prognosticate atherosclerotic disorders.[14] For that reason, we analysed the expression of CD74 in circulating cells of atherosclerotic patients and its potential association with intima-media thickness (IMT) in asymptomatic subjects.

2. Methods

2.1 Patients

The local committees on human research approved the studies, which were performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent.

Seventy consecutive patients undergoing carotid endarterectomy in Fundacion Jimenez Diaz were included. From the 70 atherosclerotic plaques (Stary stages V–VI) analysed, 27 of 70 were haemorrhagic and 34 of 70 has a lipid rich core. These plaques were fixed with paraformaldehyde and embedded in paraffin. Blood samples were also collected from 20 patients before anaesthesia on the day of endarterectomy and from 20 healthy volunteers not significantly different for sex and age. Peripheral blood mononuclear cells (PBMC) were isolated as described previously.[10]

In addition, PBMC from a group of 185 asymptomatic subjects in whom global risk assessment was performed in the course of a general health check-up by Internal Medicine Department (University Clinic of Navarra, Spain) were also studied. In all subjects, absence of history of coronary disease, stroke, or peripheral arterial disease was recorded; additional exclusion criteria were the presence of severely impaired renal function, arthritis, connective tissue diseases, alcohol abuse, or use of non-steroidal, anti-inflammatory drugs in the 2 weeks before entering the study. The following conventional cardiovascular risk factors were defined as previously described:[15] arterial hypertension and/or use of anti-hypertensive drugs; dyslipidaemia and/or use of cholesterol lowering drugs, obesity, smoking, and diabetes and/or use of pharmacological treatment. In all subjects, carotid ultrasonography was performed to determine IMT, as previously described.[15] Subjects were examined by the same two sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination. The between-observer intraclass correlation coefficient was 0.76 ($P < 0.001$) and the between subject repeatability was 0.82 ($P < 0.001$). The corresponding coefficients of variance were 5% and 10%, respectively.

2.2 RNA extraction and real-time quantitative-polymerase chain reaction

Total RNA was isolated from PBMCs, THP-1, and VSMC using TRIzol Reagent (Invitrogen); 1 μg of RNA was used to perform the reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR reactions were performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to manufacturer's protocol using the Delta-Delta Ct method. Expression levels are given as ratios to 18S. Predeveloped primer and probe assays were obtained for human 18S and CD74 from Applied Biosystems.

2.3 Immunohistochemistry

Paraffin-embedded carotid arteries were cross sectioned into 4 μm thick pieces, dewaxed, and rehydrated. Mouse monoclonal anti-human macrophage (HAM-56, DAKO), monoclonal anti α-smooth muscle actin (HHF-35, Sigma), and rabbit anti-human CD74 (FL-296, SC-20082, Santa Cruz Biotechnology) antibodies were applied. Secondary antibodies and ABComplex/HRP were added and sections were stained with 3,3′-diaminobenzidine and mounted in Pertex. For colocalization studies with VSMC and macrophages, immunohistochemistry for CD74, HAM-56, and α-actin was carried out in serial sections. For colocalization of nuclear NF-kB and CD74, immunohistochemistry for CD74 was carried out directly from the final wash of Southwestern Histochemistry as described.[10] Sirius red and Masson trichrome were performed following manufacturer's instructions.

Morphometric analysis with the Olympus semiautomatic image analysis system with Micro Image™ software (version 1.0 for windows) was performed by a pathologist (L.O.) as described.[10] Results are expressed as percentage of positive staining per mm^2.

2.4 Reagents

Recombinant human IFNγ and tumour necrosis-alfa (TNF-α) were purchased from PeproTech. Recombinant MIF was provided by R&D systems. Anti-CD74 antibody (specific for the extracellular domain of CD74, C-16, SC-5438) was from Santa Cruz Biotechnology. LDLs were obtained as

described.[16] DAPT and LY294002 were from Sigma. Z-Phe-Leu-COCHO was from Calbiochem.

2.5 Cell culture

Human aortic VSMCs (ATCC; CRL-1999) were maintained in Ham's F-12 medium supplemented with 10% FBS, penicillin–streptomycin, insulin–transferrin–sodium selenite, and 30 μg/mL endothelial cell growth supplement at 37°C in 5% CO_2. Cells were used between passages 3 and 7 as described.[17] THP-1 monocytic cell line was purchased from ATCC (CRL-1593) and cultured as described.[18]

2.6 Transfection of small-interfering RNA

VSMCs were grown to 50% confluence and transfected with a mixture composed 2.5 mol/L $CaCl_2$, 25 nmol small-interfering RNA (siRNA) (CD74 siRNA, SC-35023, or control siRNA, SC-37007), and calcium phosphate buffer in serum-free medium (Invitrogen) as previously described.[19] After 18 h, cells were washed and serum-depleted for 24 h before stimulation.

2.7 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay for NF-kB binding activity was performed with nuclear protein extracts from VSMC as described.[10] The specificity of the assay was tested with a 100-fold excess of unlabelled NF-kB consensus oligonucleotide added to the 32P-labelled probe-binding reaction.

2.8 Western blot

Equal amount of total protein was separated on 12.5% sodium dodecyl sulphate–poly-acrylamide gel electrophoresis (SDS–PAGE). Subsequently, membranes were blocked and incubated with rabbit polyclonal anti-CD74 (SC-20082, Santa Cruz Biotechnology) or mouse monoclonal anti-α-tubulin (Sigma-Aldrich). Proteins were visualized by ECL Western Blotting Detection Reagents (Amersham Biosciences) according to manufacturer's instructions.

2.9 Enzyme-linked immunosorbent assay

Soluble MCP-1 levels were measured in the supernatants of VSMC after different experimental conditions with a commercially available ELISA kit (R&D systems). Hundred microlitre of supernatant samples were assayed in parallel to known MCP-1 recombinant concentrations.

2.10 Statistical analysis

Statistics were performed using GraphPAD InStat (GraphPAD Software). *In vitro* experiments were performed at least three times. Results are expressed as mean ± SEM and were analysed by the Mann–Whitney non-parametric or Student's *t*-tests when appropriate (two-tailed, significant differences at $P < 0.05$). CD74 in PBMC of asymptomatic subjects is expressed as medians and interquartile ranges and was analysed by the Mann–Whitney *U* test. Univariate association was performed by Pearson correlation test. Multivariate linear regression analysis was conducted with carotid IMT as dependent variable, including in the model the traditional risk factors and those variables that were significant in the univariate analysis.

3. Results

3.1 CD74 is expressed in human atherosclerotic plaques

We firstly assessed the presence of CD74 in human carotid atherosclerotic plaques, analysing its localization in the different regions of the plaques: the shoulder area, characterized by a high macrophage accumulation, and the fibrous region, characterized by increased smooth muscle cell and collagen content (*Figure 1A*). Quantification of CD74 immunostaining in 70 human plaques showed an increased CD74 expression in the shoulder region in relation to the fibrous area (18.2 ± 1.3 vs. 7.8 ± 0.6% positive staining/mm^2, $P < 0.001$, *Figure 1A*). To determine the cell types contributing to CD74 expression in atherosclerotic plaques, serial sections were stained for CD74, VSMC, and macrophages. We observed that CD74 was expressed by both types of cells (*Figure 1B*). Moreover, colocalization of nuclear NF-kB and CD74 expression was noted, suggesting the potential association between these proteins *in vivo*.

3.2 CD74 is upregulated in vascular cells in response to inflammatory stimuli

We investigated the expression of CD74 in cultured vascular cells in response to several factors known to participate in atherogenesis. Western blot analysis demonstrated a time- and dose-dependent increase of CD74 protein in human VSMC (*Figure 2A*) and monocytes (*Figure 2B*) in response to IFNγ. In contrast, neither oxLDL nor TNF-α had any effect on CD74 protein levels. In addition, real-time PCR analysis showed a similar upregulation of CD74 mRNA in VSMC (*Figure 2C*) and monocytes (data not shown) in response to IFNγ.

3.3 CD74 induces MCP-1 expression in VSMC

Since CD74 has been previously related to monocyte recruitment,[6] we studied the involvement of CD74 on the chemokine MCP-1. Incubation of VSMC with an anti-CD74 antibody (which binds the CD74 extracellular domain) or IFNγ induced MCP-1 mRNA expression. In contrast, no effect was observed in the presence of the CD74 ligand, MIF, or an isotype control antibody (*Figure 3A*).

CD74 signalling involves the activation of AKT and NF-κB pathways in B cells.[2] In our studies, preincubation with the PI3/AKT-inhibitor LY294002 prevented the MCP-1 induction by anti-CD74 or IFNγ (*Figure 3B* and *C*). In addition, since intramembrane processing of CD74 is dependent on γ-secretase,[3] we used the γ-secretase/presenilin1 inhibitor DAPT in our experimental conditions. Preincubation of VSMC with DAPT abolished MCP-1 overexpression induced by anti-CD74 (*Figure 3B*). DAPT preincubation was only able to partially reverse the induction of MCP-1 by IFNγ (*Figure 3C*). Finally, since Maubach *et al.*[20] reported that IFNγ increases cathepsin S activity, we preincubated the cells with the synthetic cathepsin S inhibitor Z-Phe-Leu-COCHO,[21] which was also able to partially reverse the MCP-1 induction by IFNγ (*Figure 3C*).

3.4 CD74 siRNA decreases IFNγ-induced MCP-1 levels in VSMC

To further elucidate whether CD74 could directly participate in proinflammatory actions in vascular cells, we evaluated

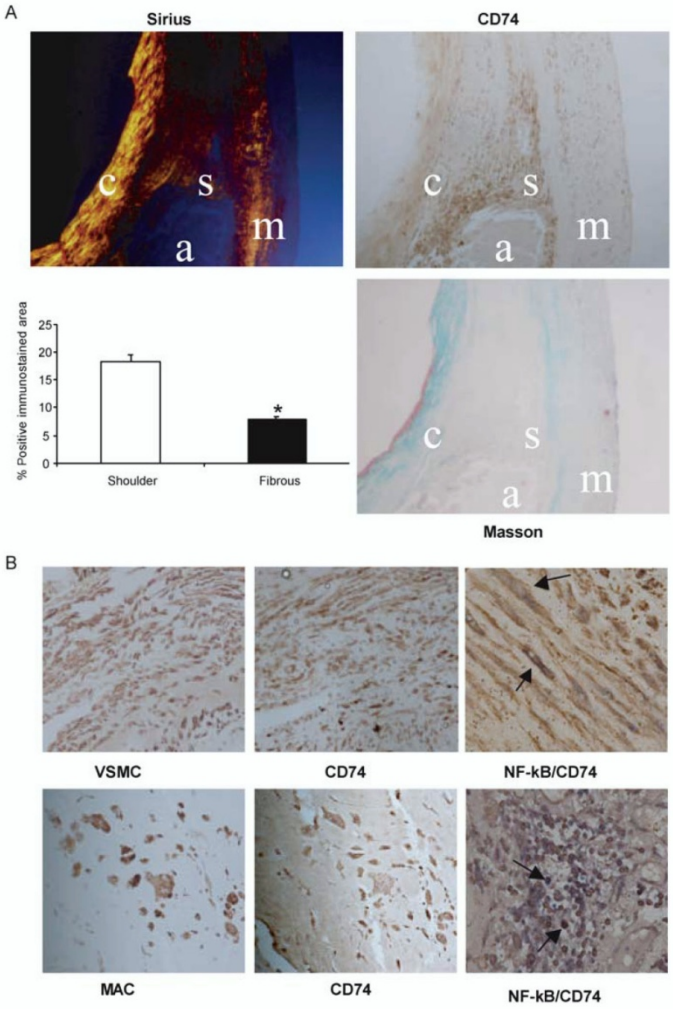


Figure 1 CD74 expression in human carotid atherosclerosis. (*A*) Expression of CD74 protein in the different regions of the plaque: shoulder (s), cap (c), atheroma (a), and media (m). Upper left: Sirius red (polarized light). Upper right: CD74 immunohistochemistry. Down right: Masson trichrome. Down left: Quantification of CD74 immunostained area in carotid atherosclerotic plaques ($n = 70$, $^*P < 0.001$). (*B*) Colocalization of CD74 and VSMC/macrophages in serial sections and CD74 expression (immunoperoxidase in brown) and NF-kB activation (phosphatase alkaline in blue) in atherosclerotic plaques. Note that no counterstaining is performed after CD74 immunostaining to avoid colour interference with blue nuclei obtained after Southwestern histochemistry.

the effect of CD74 silencing in VSMC. For that purpose, cells were transfected with a specific CD74 siRNA before IFNγ stimulation. As shown in *Figure 4A*, CD74 siRNA blocked CD74 overexpression induced by IFNγ. No effect was observed when a control siRNA was used (data not shown). Accordingly, a significant decrease in IFNγ-induced NF-kB activation and MCP-1 secretion was observed in siRNA transfected VSMC compared with non-transfected cells (*Figure 4B* and *C*). In contrast, no effect of MIF incubation on secreted MCP-1 levels was observed, similar to the results obtained at the mRNA level. Our data suggest that CD74 may participate in monocyte recruitment through the modulation of MCP-1 secretion.

3.5 CD74 expression in PBMC of human subjects

Finally, we aimed to evaluate the potential role of CD74 as a biomarker in atherosclerosis. We analysed CD74 expression in PBMCs in a training group of 20 patients with carotid atherosclerosis and 20 sex- and age-matched healthy controls. CD74 mRNA levels in PBMCs from carotid atherosclerosis patients were higher than in those of control subjects (3 ± 0.5 vs. 2 ± 0.5 AU, $P < 0.001$, *Figure 5A*). In addition, we analysed CD74 expression in a test population of 185 asymptomatic subjects in which IMT has been measured. Characteristics of the studied population are summarized

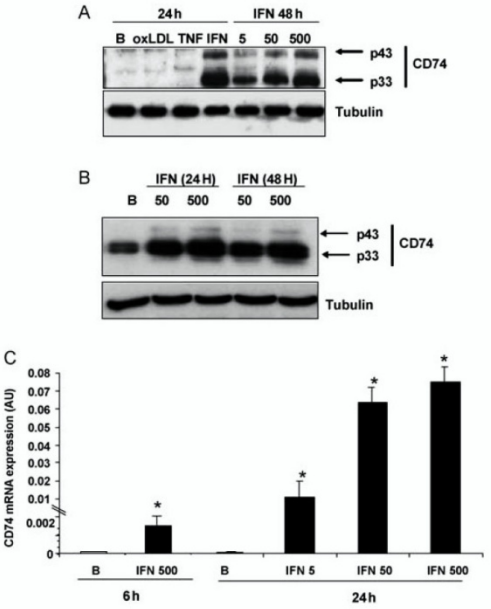


Figure 2 CD74 expression in cultured vascular cells under pro-atherogenic conditions. (*A*) Western blot analysis of CD74 in VSMCs stimulated with oxLDL (100 μg/mL), TNF-α (100 U/mL), or IFNγ (500 U/mL) for 24 h or with 5, 50, or 500 U/mL IFNγ for 48 h and (*B*) THP-1 stimulated with IFNγ (50–500 U/mL) for 24–48 h. Two bands of ~30 and 40 kDa corresponding to two different isoforms of CD74 are shown (p33 and p43). (*C*) VSMCs were stimulated with 5, 50, or 500 U/mL human IFNγ for the indicated times, and CD74 mRNA levels were determined by real-time PCR (*$P < 0.001$).

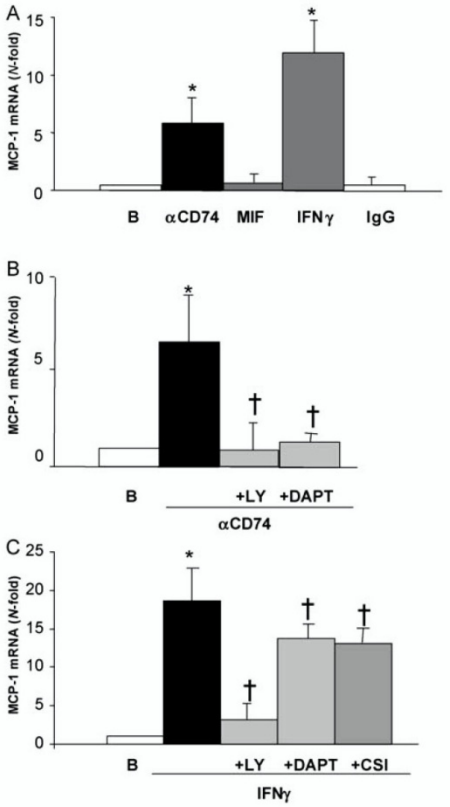


Figure 3 CD74 mediates MCP-1 expression in vascular cells. Real-time PCR of MCP-1 mRNA in (*A*) VSMC stimulated with anti-CD74 antibody (2.5 μg/mL), MIF (50 ng/mL), or IFNγ (500 U/mL) during 4 h (*$P < 0.05$). MCP-1 mRNA levels in VSMC stimulated with (*B*) anti-CD74 agonist antibody (2.5 μg/mL) during 4 h and pre-incubated with AKT-inhibitor LY294002 (LY, 50 μM) and γ-secretase inhibitor DAPT (50 μM) or stimulated with (*C*) IFNγ (500 U/mL) during 4 h and preincubated with LY (50 μM), DAPT (50 μM), or cathepsin S inhibitor (CSI, 20 nM) (*$P < 0.05$ vs. basal, †$P < 0.05$ vs. stimuli).

in *Table 1*. When we analysed tertiles of CD74 mRNA, there was a linear trend between the levels of CD74 mRNA and the increase in carotid IMT (*Figure 5B*). In addition, a univariate analysis showed a positive correlation between CD74 mRNA and IMT ($r = 0.37$; $P < 0.001$). No correlations between CD74 expression and other clinical parameters were observed (*Table 2*). Interestingly, CD74 mRNA positively correlated with MCP-1 mRNA ($r = 0.187$; $P = 0.015$). The association between CD74 and carotid IMT remained significant after adjusting for traditional risk factors (*Table 3*).

4. Discussion

It is well established that the breakdown of atherosclerotic plaques occurs more frequently at points where the fibrous cap is thinner and where there is a great amount of inflammatory cells, such as macrophages and T lymphocytes. Studies on coronary arteries of patients suffering myocardial infarction demonstrated that the rupture of atheroma usually takes place in the shoulder region,[22] an area characterized by a high-inflammatory content, NF-kB activation, and MCP-1 expression.[10,11] In this study, we observed that CD74 immunostaining is augmented in the shoulder region of human atherosclerotic plaques, and it was expressed by both macrophages and VSMC. CD74 is expressed not only by immune cells but also by fibroblasts and podocytes.[1,23] We showed colocalization of CD74 with NF-kB, suggesting that the association between both proteins could also takes place within atherosclerotic plaques. Among the different mediators involved in atherogenesis, we have observed that stimulation of vascular cells with IFNγ, but not with TNF-α or oxLDL, dose- and time-dependently induced the expression of CD74 (mRNA and protein). This is in agreement with previous papers describing CD74 upregulation by IFNγ in other cell types.[20]

The role of CD74 had been initially described as a chaperone that facilitates transport of MHC class II proteins from the endoplasmic reticulum to the Golgi complex. However, it is known that a portion of CD74 protein is expressed on the cell surface independently of class II molecules.[1] CD74 was described as a putative receptor for the inflammatory cytokine MIF.[5] Similarly, chemokine receptors (CXCR2 and CXCR4) have been recently identified as new functional receptors for MIF.[6] In this regard, inflammatory cell recruitment induced by MIF relied on CXCR2 and CD74. However,

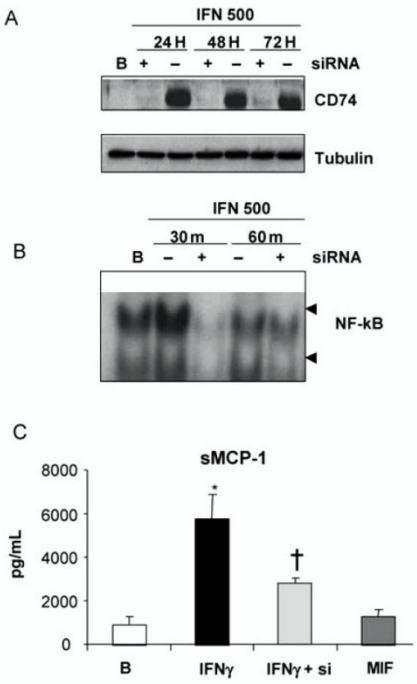


Figure 4 Effect of CD74 siRNA on IFN-γ-induced NF-kB activation and MCP-1 levels in VSMC. (*A*) Western blot showing CD74 knockdown by siRNA transfection. Cells were transfected 24 h and then stimulated 24–72 h later with IFN-γ (500 U/mL). (*B*) Representative EMSA of NF-kB activation in human VSMC transfected 24 h with CD74 siRNA and stimulated with IFN-γ (500 U/mL) for 30–60 min. (*C*) ELISA of soluble MCP-1 in conditioned media of VSMC transfected with the CD74 siRNA for 24 h followed by 24 h human IFN-γ (500 U/mL) or MIF (50 ng/mL) stimulation (*$P < 0.05$ vs. basal, B, †$P < 0.05$ vs. IFN-γ).

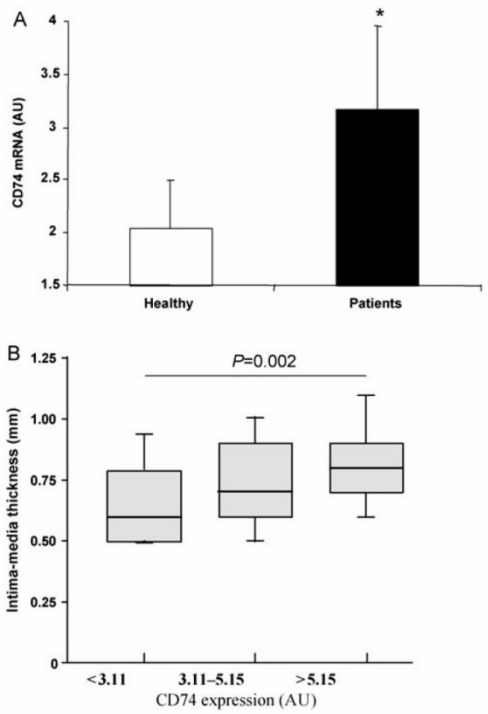


Figure 5 CD74 expression in PBMCs from human subjects. (*A*) CD74 mRNA levels in PBMCs from patients with carotid atherosclerosis ($n = 20$) and from healthy controls (*$P < 0.001$, real-time PCR). (*B*) Carotid IMT according to the tertiles of CD74 mRNA expression in PBMC from asymptomatic subjects ($n = 185$, $P < 0.001$, real-time PCR). Boxes represent 25th and 75th percentiles; line within boxes, median. Error bars mark 10th and 90th percentile.

blocking CD74 in $MIF^{-/-}$ mice prevented inflammatory cell recruitment, suggesting that CD74 could participate, independently of MIF, in atherogenic cell recruitment.[6] In this respect, the role of cell-surface CD74 and the identity of its natural ligand in VSMC and other cells are still unknown. Stimulation of CD74 with an anti-CD74 antibody, which binds the CD74 extracellular domain, results in the activation of AKT and NF-κB pathways in B cells.[2] We have observed that both anti-CD74 and IFN-γ, but not MIF, induced the expression of the NF-kB-regulated gene MCP-1 in VSMC, and this effect was prevented by an AKT-inhibitor. NF-kB is activated by the intracellular domain of CD74, which is released from the membrane after γ-secretase cleavage.[3] We have shown that the γ-secretase/presenilin1 inhibitor DAPT blocks anti-CD74-induced MCP-1 expression in VSMC. In addition, both γ-secretase and cathepsin S inhibitors partially reduced MCP-1 induction by IFN-γ. In this regard, CD74 silencing significantly decreased NF-kB activation and MCP-1 secretion induced by IFN-γ. In contrast, no effect of MIF incubation on secreted MCP-1 levels was observed. Our results suggest a novel mechanism by which IFN-γ induces MCP-1 expression and secretion implicating CD74 (*Figure 6*). However, since CD74 silencing was not able to completely block this effect, we cannot discard that other mechanisms of MCP-1 induction by IFN-γ could also take place. In this regard, cellular responses to IFN-γ such as MCP-1 expression are mainly mediated by JAK–STAT pathway.[24] Whether CD74 signalling is linked to STAT activation, as previously shown for AKT, is unknown at present and will require further investigations.

There are several potential explanations for these results. First, IFN-γ may directly induce CD74 intramembrane cleavage and release to the nucleus, as recently shown for IL-8,[13] or indirectly via Cathepsin S upregulation[20] or by other yet unknown mechanisms. Secondly, CD74 may recruit other proteins to its proximity to initiate signalling cascades. In this respect, CD74 can interact with CD44,[25] angiotensin AT1-receptor[26] or nitric-oxide synthase 2.[27] In addition, ectodomain shedding is regulated by PKC activity[28] and signalling events initiated by IFN-γ involve activation of PKC and calcium–calmodulin protein kinases. We have observed that blocking PKC with staurosporin also decreases IFN-γ-induced MCP-1 expression in our experimental conditions (unpublished results). Thirdly, CD74 processing could be ligand dependent or constitutive, as shown for other RIP proteins.[29]

Table 1 Baseline clinical characteristics of the studied population

Total population ($n = 185$)	
Age, year	54.1 ± 0.8
Gender, male/female	153/32
BMI, kg/m^2	28.9 ± 0.3
SBP, mmHg	130.6 ± 1.4
DBP, mmHg	82.6 ± 0.7
Arterial hypertension, yes/no	104/81
Diabetes mellitus, yes/no	23/162
Obese, yes/no	66/119
Glucose, mg/dL	105.4 ± 2.2
Total cholesterol, mg/dL	220.0 ± 3.0
HDL cholesterol	45.6 ± 0.8
LDL cholesterol	148.5 ± 2.8
Triglycerides, mg/dL	129.3 ± 5.3
C-reactive protein, mg/dL	0.39 ± 0.04
Fibrinogen, mg/dL	282.9 ± 4.7
vWF, %	116.5 ± 3.5
eGFR, mL/min/1.73 m^2	87.0 ± 1.35
CD74, AU	4.38 ± 0.14
MCP-1, AU	2.68 ± 0.12
Left carotid IMT, mm	0.74 ± 0.01

Values are expressed as mean ± SEM and number of subjects. BMI, body mass index; DBP, diastolic blood pressure; IMT, intima-media thickness; SBP, systolic blood pressure; vWF, von Willebrand factor; eGFR, estimated glomerular filtration rate; MCP-1, monocyte chemoattractant protein-1.

Table 2 Correlation coefficients of left carotid intima-media thickness and CD74 with clinical and laboratory parameters in the studies population

	CD74		IMT	
	r	P-value	r	P-value
Age, year	0.047	0.525	0.336	<0.001
BMI, kg/m^2	0.012	0.869	0.076	0.305
SBP, mmHg	0.053	0.475	0.162	0.028
DBP, mmHg	0.028	0.704	0.130	0.077
Glucose, mg/dL	0.086	0.246	0.060	0.589
Total cholesterol, mg/dL	0.056	0.445	0.040	0.589
HDL cholesterol	0.062	0.404	0.012	0.873
LDL cholesterol	0.048	0.520	0.025	0.735
Triglycerides, mg/dL	−0.028	0.702	0.034	0.647
C-reactive protein, mg/dL	−0.098	0.196	−0.099	0.194
Fibrinogen, mg/dL	−0.136	0.067	−0.054	0.480
vWF, %	−0.135	0.700	−0.026	0.723
eGFR, mL/min/1.73 m^2	0.830	0.290	−0.120	0.874
MCP-1, AU	0.187	0.014	0.044	0.574
CD74, AU	–	–	0.374	<0.001
Left carotid IMT, mm	0.374	<0.001	–	–

Correlations and P-values from Pearson correlation coefficient.

Table 3 Multiple linear regression analysis with carotid intima-media thickness as dependent variable

Independent variables	β	P-value	Partial r^2 (%)
CD74, AU	0.031	<0.001	14.6
Age, year	0.006	<0.001	10.4
Gender, male/female	−0.100	0.002	6.3
Smoking, n/y	0.065	0.011	2.6
BMI, kg/m^2	0.001	0.620	0.0
SBP, mmHg	0.000	0.491	0.1
Glucose, mg/dL	0.000	0.286	0.5
Total cholesterol, mg/dL	0.000	0.650	0.0
C-reactive protein, mg/dL	−0.039	0.105	1.1
MCP-1, AU	−0.003	0.682	0.0

β, regression coefficient; R^2, partial correlation coefficient after adjustment. Adjusted for age, gender, smoking, BMI, systolic blood pressure, total cholesterol, glucose, C-reactive protein, and MCP-1. R^2 for the total population was 35.4%.

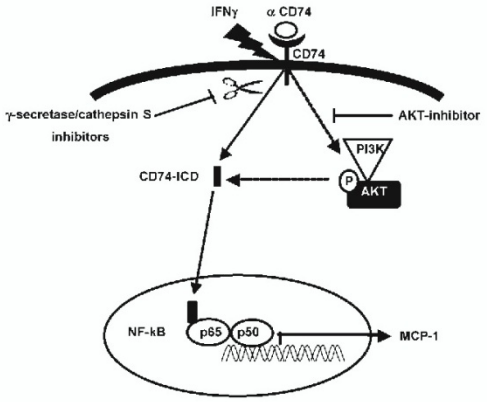


Figure 6 Schematic representation of the potential mechanisms by which CD74 is involved in MCP-1 induction. CD74 can be activated by anti-CD74 agonist or by IFNγ and this could lead to CD74 processing by different proteases (g-secretase/cathepsin S), a process which is regulated by AKT/PI3K inhibitors. Following CD74 processing, CD74 intracellular domain (CD74-ICD) can translocate to the nucleus and activate NF-kB, favouring MCP-1 release.

Being able to predict who is at risk of an acute thrombotic event is at present one of the major challenges of cardiovascular medicine. Increases in the thickness of the intima and media of the carotid artery, as measured non-invasively by ultrasonography, are directly associated with an increased risk of myocardial infarction and stroke.[30,31] While non-invasive imaging techniques are being investigated to improve characterization of the size and morphology of the atherosclerotic plaques, other field of growing interest is the search of blood diagnostic/prognostic biomarkers. In this regard, several inflammatory proteins involved in the pathophysiology of atherothrombosis have been evaluated. Among them, NF-kB activity is elevated in circulating cells from patients with unstable angina,[32] patients with carotid stenosis[10] and during acute coronary syndromes.[33] These studies suggest that circulating monocytes are activated before entering the vascular wall. In a recent study, the changes in the gene expression profile of primary monocytes after constitutive adhesion to endothelial cells have shown an upregulation of CD74, suggesting that CD74 overexpression could be an early biomarker of the initiation of the

differentiation program of monocytes into macrophages.[34] In our pilot study, we have observed that CD74 expression is significantly increased in PBMCs from carotid atherosclerosis patients compared with healthy individuals. Furthermore, we have shown that CD74 expression correlates with IMT in a test population of 185 asymptomatic subjects. Interestingly, correlation of CD74 with IMT remained significant after adjusting for some potentially confounding classical risk factors indicating that CD74 levels could be an independent biomarker of atherosclerosis. Future studies are needed to confirm the potential role of CD74 as a biomarker of sub-clinical atherosclerosis.

On the whole, our data suggest a role for CD74 in the inflammatory cascade during atherogenesis. Its biological functions, its presence in vulnerable regions of human plaques, and its association with surrogate markers such as IMT could implicate CD74 as a potential therapeutic target in atherosclerosis.

Conflict of interest: none declared.

Funding

This work was supported by the Spanish Ministerio de Ciencia y Tecnología (SAF2007/63648), Educación y Ciencia (SAF2007-62533), Fundacion Ramon Areces, CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035; RD06/0014/0008) REDinREN/RD06/0016 and European Network (HEALTH F2-2008-200647), and European Union (InGenious HyperCare, grant LSHM-CT-2006-037093). Programa Intensificación Actividad Investigadora Sistema Nacional de Salud ISCIII/CM to A.O. This project was also funded through the agreement between the Foundation for Applied Medical Research (FIMA) and 'UTE project CIMA'.

References

1. Henne C, Schwenk F, Koch N, Moller P. Surface expression of the invariant chain (CD74) is independent of concomitant expression of major histocompatibility complex class II antigens. *Immunology* 1995;**84**:177–182.
2. Starlets D, Gore Y, Binsky I, Haran M, Harpaz N, Shvidel L *et al.* Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. *Blood* 2006;**107**:4807–4816.
3. Becker-Herman S, Arie G, Medvedovsky H, Kerem A, Shachar I. CD74 is a member of the regulated intramembrane proteolysis-processed protein family. *Mol Biol Cell* 2005;**16**:5061–5069.
4. Brown MS, Ye J, Rawson RB, Goldstein JL. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 2000;**100**:391–398.
5. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J *et al.* MIF signal transduction initiated by binding to CD74. *J Exp Med* 2003;**197**:1467–1476.
6. Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR *et al.* MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med* 2007;**13**:587–596.
7. Beswick EJ, Das S, Pinchuk IV, Adegboyega P, Suarez G, Yamaoka Y *et al.* *Helicobacter pylori*-induced IL-8 production by gastric epithelial cells up-regulates CD74 expression. *J Immunol* 2005;**175**:171–176.
8. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* 2006;**86**:515–581.
9. Takahashi M, Masuyama J, Ikeda U, Kasahara T, Kitagawa S, Takahashi Y *et al.* Induction of monocyte chemoattractant protein-1 synthesis in human monocytes during transendothelial migration *in vitro*. *Circ Res* 1995;**76**:750–757.
10. Martín-Ventura JL, Ortego M, Esbrit P, Hernández-Presa MA, Ortega L, Egido J. Possible role of parathyroid hormone-related protein as a proinflammatory cytokine in atherosclerosis. *Stroke* 2003;**34**:1783–1789.
11. Martín-Ventura JL, Blanco-Colio LM, Muñoz-García B, Gómez-Hernández A, Arribas A, Ortega L *et al.* NF-kappaB activation and Fas ligand overexpression in blood and plaques of patients with carotid atherosclerosis: potential implication in plaque instability. *Stroke* 2004;**35**:458–463.
12. Maier M, Wutzler S, Bauer M, Trendafilov P, Henrich D, Marzi I. Altered gene expression patterns in dendritic cells after severe trauma: implications for systemic inflammation and organ injury. *Shock* 2008;**30**:344–351.
13. Binsky I, Haran M, Starlets D, Gore Y, Lantner F, Harpaz N *et al.* IL-8 secreted in a macrophage migration-inhibitory factor- and CD74-dependent manner regulates B cell chronic lymphocytic leukemia survival. *Proc Natl Acad Sci USA* 2007;**104**:13408–13413.
14. Aukrust P, Halvorsen B, Yndestad A, Ueland T, Oie E, Otterdal K *et al.* Chemokines and cardiovascular risk. *Arterioscler Thromb Vasc Biol* 2008;**28**:1909–1919.
15. Zalba G, Fortuño A, Orbe J, San José G, Moreno MU, Belzunce M *et al.* Phagocytic NADPH oxidase-dependent superoxide production stimulates matrix metalloproteinase-9: implications for human atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007;**27**:587–593.
16. Martin-Ventura JL, Blanco-Colio LM, Aparicio C, Ortega L, Esbrit P, Egido J. LDL induces parathyroid hormone-related protein expression in vascular smooth muscle cells: modulation by simvastatin. *Atherosclerosis* 2008;**198**:264–271.
17. Muñoz-García B, Martín-Ventura JL, Martínez E, Sánchez S, Hernández G, Ortega L *et al.* Fn14 is upregulated in cytokine-stimulated vascular smooth muscle cells and is expressed in human carotid atherosclerotic plaques: modulation by atorvastatin. *Stroke* 2006;**37**:2044–2053.
18. Gómez-Hernández A, Martín-Ventura JL, Sánchez-Galán E, Vidal C, Ortego M, Blanco-Colio LM *et al.* Overexpression of COX-2, Prostaglandin E synthase-1 and prostaglandin E receptors in blood mononuclear cells and plaque of patients with carotid atherosclerosis: regulation by nuclear factor-kappaB. *Atherosclerosis* 2006;**187**:139–149.
19. Martin-Ventura JL, Nicolas V, Houard X, Blanco-Colio LM, Leclercq A, Egido J *et al.* Biological significance of decreased HSP27 in human atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006;**26**:1337–1343.
20. Maubach G, Lim MC, Kumar S, Zhuo L. Expression and upregulation of cathepsin S and other early molecules required for antigen presentation in activated hepatic stellate cells upon IFN-gamma treatment. *Biochim Biophys Acta* 2007;**1773**:219–231.
21. Taleb S, Cancello R, Clément K, Lacasa D. Cathepsin s promotes human preadipocyte differentiation: possible involvement of fibronectin degradation. *Endocrinology* 2006;**147**:4950–4959.
22. Kaartinen M, Penttilä A, Kovanen PT. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* 1994;**90**:1669–1678.
23. Sanchez-Niño MD, Sanz AB, Ihalmo P, Lassila M, Holthofer H, Mezzano S *et al.* The MIF receptor CD74 in diabetic podocyte injury. *J Am Soc Nephrol* 2009;**20**:353–362.
24. Ortiz-Muñoz G, Martin-Ventura JL, Hernandez-Vargas P, Mallavia B, Lopez-Parra V, Lopez-Franco O *et al.* Suppressors of cytokine signaling modulate JAK/STAT-mediated cell responses during atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009;**29**:525–531.
25. Shi X, Leng L, Wang T, Wang W, Du X, Li J *et al.* CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity* 2006;**25**:595–606.
26. Szaszak M, Chen HD, Chen HC, Baukal A, Hunyady L, Catt K. Identification of the invariant chain (Ii/CD74) as an angiotensin AT1-receptor-interacting protein. *J Endocrinol* 2008;**199**:165–176.
27. Huang D, Cai DT, Chua RY, Kemeny DM, Wong SH. Nitric-oxide synthase 2 interacts with CD74 and inhibits its cleavage by caspase during dendritic cell development. *J Biol Chem* 2008;**283**:1713–1722.
28. Schlöndorff J, Blobel CP. Metalloprotease-disintegrins: modular proteins capable of promoting cell–cell interactions and triggering signals by protein-ectodomain shedding. *J Cell Sci* 1999;**112**:3603–3617.
29. Zou Z, Chung B, Nguyen T, Mentone S, Thomson B, Biemesderfer D. Linking receptor-mediated endocytosis and cell signaling: evidence for regulated intramembrane proteolysis of megalin in proximal tubule. *J Biol Chem* 2004;**279**:34302–34310.
30. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam study. *Circulation* 1997;**128**:262–269.
31. Nichols WW, Pepine CJ, O'Rourke MF. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke. *N Engl J Med* 1999;**340**:1762–1763.

32. Ritchie ME. Nuclear factor-kappaB is selectively and markedly activated in humans with unstable angina pectoris. *Circulation* 1998;**98**: 1707–1713.
33. Liuzzo G, Santamaria M, Biasucci LM, Narducci M, Colafrancesco V, Porto A *et al.* Persistent activation of nuclear factor kappa-B signaling pathway in patients with unstable angina and elevated levels of C-reactive protein. Evidence for a direct proinflammatory effect of azide and lipopolysaccharide-free C-reactive protein on human monocytes via nuclear factor kappa-B activation. *J Am Coll Cardiol* 2007;**49**: 185–194.
34. Thomas-Ecker S, Lindecke A, Hatzmann W, Kaltschmidt C, Zänker KS, Dittmar T. Alteration in the gene expression pattern of primary monocytes after adhesion to endothelial cells. *Proc Natl Acad Sci USA* 2007; **104**:5539–5544.

http://www.kidney-international.org original article

Treatment with amlodipine and atorvastatin has additive effect on blood and plaque inflammation in hypertensive patients with carotid atherosclerosis

José L. Martín-Ventura[1,4], Begoña Muñoz-Garcia[1,4], Luis M. Blanco-Colio[1], Antonio Martín-Conejero[2], Julio Madrigal-Matute[1], Melina Vega[3], Luis Ortega[2], Javier Serrano[2] and Jesús Egido[1]

[1]The Renal and Vascular Research Lab, Fundacion Jimenez Diaz, Autonoma University, Madrid, Spain; [2]Vascular Surgery and Anatomy Pathology Services, Hospital Clínico de San Carlos, Universidad Complutense, Madrid, Spain and [3]Vascular Surgery, Hospital de Galdakao, Vizcaya, Spain

Since previous studies have reported a beneficial effect of amlodipine and atorvastatin treatment in experimental atherosclerosis, we aimed to investigate the effect of the combination of both drugs on blood and plaque inflammation in patients with carotid stenosis. For that purpose, twenty six hypertensive patients undergoing carotid endarterectomy were randomized to receive either atorvastatin 20 mg/day alone (ATV, n = 12) or in combination with amlodipine 20 mg/day (ATV + AML, n = 14) before scheduled carotid endarterectomy. At the end of follow-up (4-6 weeks), there was a significant decrease in total and LDL-cholesterol levels, but not in blood pressure levels. In contrast, decreased MCP-1 plasma levels, NF-κB activation (EMSA) and MCP-1 mRNA expression (quantitative PCR) was only observed in blood from ATV + AML treated-patients. Moreover, carotid atherosclerotic plaques from ATV + AML group demonstrated a significant reduction in macrophage infiltration in relation to ATV group (immunohistochemistry). Our results suggest that combined treatment with atorvastatin and amlodipine decreases inflammatory status of atherosclerotic patients more than atorvastatin treatment alone, suggesting that co-administration of both drugs could have beneficial additive effects.

Kidney International (2008) **74** (Suppl 111), S71–S74; doi:10.1038/ki.2008.521

KEYWORDS: carotid artery; atherosclerosis; inflammation; amlodipine; atorvastatin

Correspondence: *Jesús Egido, Renal and Vascular Research Lab, Division of Nephrology and Hypertension, Fundación Jiménez Díaz, Avenida Reyes Católicos 2, Madrid 28040, Spain. E-mail: jegido@fjd.es*

[4]These authors contributed equally to this work.

Hypertension and hypercholesterolemia are important modifiable risk factors for cardiovascular disease. However, treatment and control of combined hypertension and hypercholesterolemia are suboptimal.[1] Calcium channel blockers (CCBs) have been used for decades to treat hypertension, but it has also been suggested that they interfere with the progression of atherosclerotic disease.[2] Among these drugs, amlodipine reduced carotid intima-media thickness progression and the incidence of unstable angina and revascularization in patients with coronary artery disease (CAD).[3] More recently, amlodipine was able to slow the progression of atherosclerosis and reduce cardiovascular events in patients with CAD and normal blood pressure (BP).[4] These effects could be due to the special profile of amlodipine, which includes antioxidant, antiproliferative, and anti-inflammatory properties.[5,6] Statins are powerful drugs widely used in the treatment of cardiovascular diseases. Apart from their known hypolipemic properties, they have been reported to have pleiotropic effects, such as anti-inflammatory and immunomodulatory actions.[7] In this respect, we have shown earlier that atorvastatin (80 mg/day) decreased nuclear factor-κB (NF-κB) activation and monocyte chemoattractant protein-1 (MCP-1) expression in peripheral blood mononuclear cells, as well as macrophage infiltration in patients with carotid atherosclerosis.[8] Given that statins and CCBs have different mechanisms of action, it is conceivable that they may have an additive or synergic effect not only on new plaque formation but also on inhibiting the progression of established lesions.[9] Indeed, this synergic effect of lipid-lowering therapy and CCBs on human coronary atherosclerosis has been reported in the Regression Growth Evaluation Statin Study (REGRESS) trial.[10] The beneficial effect of combining CCBs with statins has been replicated in transgenic atherosclerotic mice, where treatment with amlodipine and atorvastatin produced an additional reduction of atherosclerosis compared with that observed with either amlodipine or atorvastatin alone.[11] As experimental studies have shown that statins and CCBs have

additive anti-atherosclerotic effects, we decided to analyze the effect of atorvastatin in combination with amlodipine on inflammatory parameters both in blood and in atherosclerotic plaques of hypertensive patients with carotid stenosis.

RESULTS

Effect of atorvastatin + amlodipine on lipid and BP levels

Clinical data and baseline BP and lipid levels, as well as concomitant treatments, were well balanced between both groups. At the end of the study (4–6 weeks), total and low-density lipoproteins cholesterol levels were decreased in both groups, whereas no significant changes were observed in high-density lipoproteins and triglyceride values or in BP levels (Table 1).

Effect of atorvastatin + amlodipine in blood inflammatory status

Patients treated with atorvastatin + amlodipine displayed a reduction in MCP-1 plasma levels as compared with their baseline values (137 ± 39 vs 111 ± 38 pg/ml, $P = 0.01$). Although there was a trend, no significant differences for MCP-1 were observed in atorvastatin-treated patients (146 ± 43 vs 125 ± 62 pg/ml, $P = 0.1$). Furthermore, atorvastatin + amlodipine-treated patients had decreased levels of MCP-1 when compared with the atorvastatin-treated group (Table 1).

Peripheral blood mononuclear cells from patients who received treatment with atorvastatin + amlodipine showed diminished NF-κB activation when compared with the atorvastatin-treated patients (0.73 ± 0.25 vs 1.34 ± 0.31 times vs day 0, $P < 0.05$) as evidenced by electrophoretic mobility shift assay. In addition, MCP-1 mRNA expression was reduced in peripheral blood mononuclear cells from patients treated with atorvastatin + amlodipine compared with the atorvastatin-treated group (0.94 ± 0.26 vs 1.53 ± 0.44 times vs day 0, $P < 0.05$) as assessed by quantitative PCR.

Effect of atorvastatin + amlodipine on plaque composition

Carotid atherosclerotic plaques from patients treated with atorvastatin + amlodipine displayed less macrophage infiltration than those of the atorvastatin-treated group (18 ± 9 vs $23 \pm 11\%$ of positive staining per mm^2, $P < 0.05$, Figure 1). In contrast, no significant differences were observed either in the number of VSMC or in the total number of cells between both groups.

DISCUSSION

In the last years, there has been increasing evidence suggesting that the treatment of cardiovascular risk factors must be performed on a global rather than on a separate approach, because they have additive effects and share common pathways leading to atherothrombosis.[12] A large number of clinical trials have shown that treatment of either hypercholesterolemia or hypertension leads to a reduction in the incidence of cardiovascular events.[13–16] Among them, in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)-BPLA (Blood Pressure-Lowering Arm), an optimal prevention of cardiovascular events was reached in patients randomized to atorvastatin and the amlodipine/perindopril treatment, with a reduction of 48% in the risk of fatal myocardial infarction and non-fatal coronary heart disease and of 44% in the incidence of stroke. However, the potential mechanisms underlying these beneficial effects have not been fully elucidated.

Earlier experimental studies have shown that the addition of amlodipine to atorvastatin is able to enhance the beneficial effect of atorvastatin on plaque progression.[11] Among the proposed vasculoprotective effects of this drug combination are their capacity to reverse endothelial dysfunction or to decrease oxidative stress.[17] In addition, different studies in hypertensive hyperlipidemic patients have shown that the combination of atorvastatin and amlodipine has additive effects in the improvement of arterial compliance and in the fibrinolytic balance, early markers of vascular damage and atherosclerosis.[18,19] In this respect, we have shown that the addition of amlodipine to atorvastatin provides a higher effect on decreasing blood inflammatory molecules, as well as on reducing plaque macrophage infiltration, a marker of progression and instability of atherosclerotic lesions. We did not observe a significant decrease on BP levels after amlodipine treatment, suggesting that the beneficial effects observed were independent of the antihypertensive effects of amlodipine, in agreement with earlier studies.[11] In this respect, other mechanisms directly related to amlodipine treatment should also be involved. Amlodipine is able to decrease the expressions of adhesion molecules (for example, vascular cell adhesion molecule-1) or chemokines (e.g. MCP-1) in the aorta of atherosclerotic mice[6] and to modify the binding of monocytes to the endothelium.[20] Collectively, these studies support the clinical antiatherosclerotic advantages of the combination of both CCBs and statins and, in particular, of atorvastatin with amlodipine beyond their established antihyperlipemic and antihypertensive modes of action.

The major limitation of this study is the sample size. At present, an increasing number of patients with carotid atherosclerosis receive a stent instead of having an endarterectomy. Moreover, only asymptomatic patients were included for ethical considerations and some of them were excluded because of the high doses of statins that they were receiving. Despite these limitations, this study provides important new findings regarding the co-administration of amlodipine and atorvastatin to hypertensive patients with carotid stenosis. Larger and perhaps longer-term studies in hypertensive patients with carotid atherosclerosis are essential to further explore these potential benefits. In summary, different studies support that a combined treatment of hypertensive patients with both CCBs and statins could promote a higher reduction in their global cardiovascular risk profile and associated mortality. In this respect, our results suggest that combined treatment with atorvastatin and amlodipine

Table 1 | Lipid, blood pressure, and inflammatory plasma levels

	Atorvastatin (day 0)	Atorvastatin (1 month)	At+Am (day 0)	At+Am (1 month)
Cholesterol	177 ± 39	134 ± 26*	181 ± 44	146 ± 36**
LDL-c	104 ± 31	67 ± 14*	108 ± 30	74 ± 23*
Triglycerides	131 ± 51	108 ± 46	138 ± 83	111 ± 48
HDL-c	49 ± 11	44 ± 9	47 ± 9	49 ± 7
SBP	147 ± 20	142 ± 16	147 ± 20	142 ± 14
DBP	76 ± 14	75 ± 11	77 ± 15	75 ± 10
MCP-1	146 ± 43	125 ± 62	137 ± 39	111 ± 38*

Am, amlodipine; At, atorvastatin; DBP, diastolic blood pressure; HDL-c, high-density lipoproteins cholesterol; LDL-c, low-density lipoproteins cholesterol; MCP-1, monocyte chemoattractant protein-1; SBP, systolic blood pressure.
Values are displayed as mean ± s.e.m. Cholesterol, LDL-c, tryglicerides, and HDL-c units are mg per 100 ml. BP levels are in mm Hg. MCP-1 levels are in pg/ml. *$P<0.01$ vs day 0 and **$P<0.05$ vs day 0. Differences between basal lipid and inflammatory levels of both treatment groups were not statistically significant.

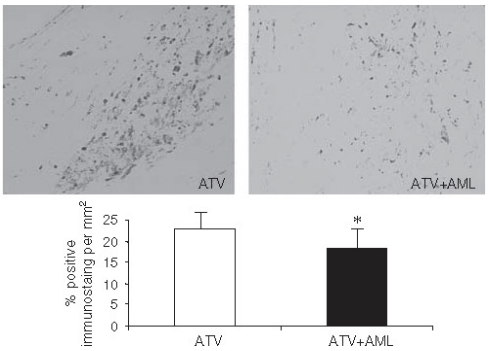


Figure 1 | Macrophage infiltration in human atherosclerotic plaques. Macrophage infiltration in carotid atherosclerotic plaques was reduced in the atorvastatin + amlodipine (ATV + AML) group in relation to atorvastatin (ATV) (immunohistochemistry).

decreases the inflammatory status of atherosclerotic patients more than atorvastatin alone, suggesting that co-administration of both drugs could have beneficial additive effects.

MATERIALS AND METHODS

Patients with asymptomatic carotid stenosis >70%, with low-density lipoproteins cholesterol >130 mg per 100 ml and with DBP >90 and/or SBP >140 mm Hg, were recruited (70 ± 7 years, 21men and 5women, 34% hyperlipidemic, 26% diabetic). At the moment of surgical indication, informed consent was obtained, BP was measured, blood was extracted for the analysis of lipid and inflammatory parameters, and patients were randomized to receive either atorvastatin 20 mg/day alone ($n-12$) or in combination with amlodipine 20 mg/day ($n-14$) until scheduled carotid endarterectomy was performed (4–6 weeks). At surgery, BP was measured, and blood samples and the atherosclerotic plaques were collected. The study was approved by the Ethical Committee of the Institutions. Total cholesterol, low-density lipoproteins, triglycerides, and high-density lipoproteins were measured by enzymatic assays (Sigma Diagnostics, St Louis, MO, USA). MCP-1 plasma levels were determined by ELISA (R&D Systems, Minneapolis, MN, USA). A volume of 50 ml of blood was drawn from the patients at random and before surgery. Peripheral blood mononuclear cells were obtained as described[8] and later resuspended for nuclear protein or RNA extraction. NF-κB activation was analyzed by Electrophoretic Mobility Shift Assay.[8] MCP-1 mRNA expression was assayed by quantitative PCR and values were normalized by 18S levels.[8] Results (arbitrary units) were expressed as times vs day 0 to normalize interpatient variability. Carotid arteries were collected, stored in paraformaldehyde for 24 h and later in ethanol until paraffin-embedded. Primary antibodies (monoclonal anti-human macrophages HAM-56 and anti-α-smooth muscle actin HHF-35) were applied.[8] Morphometric analysis with the Olympus semiautomatic image analysis system with Micro Image software (version 1.0 for Windows) was performed by a pathologist (LO) blinded to the group to which the atherosclerotic plaques belonged.[8] Results are expressed as the percentage of positive staining per mm^2. Statistical analysis was performed with GraphPAD InStat (GraphPAD Software). Data were presented as mean ± s.e.m. Lipid levels and ELISA data were analyzed by the paired Wilcoxon test. Electrophoretic mobility shift assay, quantitative PCR, and immunohistochemistry data at the end of the study were analyzed by the Mann–Whitney test (significant differences at two-tailed $P<0.05$).

DISCLOSURE

The authors have declared no financial interests.

ACKNOWLEDGMENTS

This study was supported by Pfizer-Spain, the Spanish Ministerio de Ciencia y Tecnología (SAF SAF2007/63648), CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, and Redes RECAVA (RD06/0014/0035).

REFERENCES

1. Wong ND, Lopez V, Tang S *et al.* Prevalence, treatment, and control of combined hypertension and hypercholesterolemia in the United States. *Am J Cardiol* 2006; **98**: 204 208.
2. Boden WE, van Gilst WH, Scheldewaert RG *et al.* Diltiazem in acute myocardial infarction treated with thrombolytic agents: a randomised placebo-controlled trial. Incomplete Infarction Trial of European Research Collaborators Evaluating Prognosis post-Thrombolysis (INTERCEPT). *Lancet* 2000; **355**: 1751 1756.
3. Pitt B, Byington RP, Furberg CD *et al.* Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. PREVENT Investigators. *Circulation* 2000; **102**: 1503 1510.
4. Nissen SE, Tuzcu EM, Libby P *et al.* Effect of antihypertensive agents on cardiovascular events in patients with coronary disease and normal blood pressure: the CAMELOT study: a randomized controlled trial. *JAMA* 2004; **292**: 2217 2225.

5. Zhou MS, Jaimes EA, Raij L. Inhibition of oxidative stress and improvement of endothelial function by amlodipine in angiotensin II-infused rats. *Am J Hypertens* 2004; **17**: 167–171.
6. Yoshii T, Iwai M, Li Z *et al.* Regression of atherosclerosis by amlodipine via anti-inflammatory and anti-oxidative stress actions. *Hypertens Res* 2006; **29**: 457–466.
7. Blanco-Colio LM, Tuñón J, Martín-Ventura JL *et al.* Anti-inflammatory and immunomodulatory effects of statins. *Kidney Int* 2003; **63**: 12–23.
8. Martín-Ventura JL, Blanco-Colio LM, Gómez-Hernández A *et al J.* Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. *Stroke* 2005; **36**: 1796–1800.
9. Mason RP. Scientific rationale for combination of a calcium channel antagonist and an HMG-CoA reductase inhibitor: a new approach to risk factor management. *Drugs* 2008; **68**: 885–900.
10. Jukema JW, Zwinderman AH, van Boven AJ *et al.* Evidence for a synergistic effect of calcium channel blockers with lipid-lowering therapy in retarding progression of coronary atherosclerosis in symptomatic patients with normal to moderately raised cholesterol levels. The REGRESS Study Group. *Arterioscler Thromb Vasc Biol* 1996; **16**: 425–430.
11. Trion A, de Maat M, Jukema W *et al.* Anti-atherosclerotic effect of amlodipine, alone and in combination with atorvastatin, in APOE*3-Leiden/hCRP transgenic mice. *J Cardiovasc Pharmacol* 2006; **47**: 89–95.
12. Tuñón J, Martín-Ventura JL, Blanco-Colio LM *et al.* Common pathways of hypercholesterolemia and hypertension leading to atherothrombosis: the need for a global approach in the management of cardiovascular risk factors. *Vasc Health Risk Manag* 2007; **3**: 521–526.
13. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* 2002; **360**: 7–22.
14. Dahlof B, Sever PS, Poulter NR *et al.* Prevention of cardiovascular events with an antihypertensive regimen of amlodipine adding perindopril as required versus atenolol adding bendroflumethiazide as required, in the Anglo-Scandinavian Cardiac Outcomes Trial-Blood Pressure Lowering Arm (ASCOT-BPLA): a multicentre randomised controlled trial. *Lancet* 2005; **366**: 895–906.
15. Julius S, Kjeldsen SE, Weber M *et al.* VALUE Trial Group. Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine: the VALUE randomised trial. *Lancet* 2004; **363**: 2022–2031.
16. Sever PS, Dahlof B, Poulter NR *et al.* Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial—Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet* 2003; **361**: 1149–1158.
17. Mason RP, Kubant R, Heeba G *et al.* Synergistic effect of amlodipine and atorvastatin in reversing LDL-induced endothelial dysfunction. *Pharm Res* 2008; **25**: 1798–1806.
18. Leibovitz E, Beniashvili M, Zimlichman R *et al.* Treatment with amlodipine and atorvastatin have additive effect in improvement of arterial compliance in hypertensive hyperlipidemic patients. *Am J Hypertens* 2003; **16**: 715–718.
19. Fogari R, Derosa G, Lazzari P *et al.* Effect of amlodipine-atorvastatin combination on fibrinolysis in hypertensive hypercholesterolemic patients with insulin resistance. *Am J Hypertens* 2004; **17**: 823–827.
20. Eickelberg O, Roth M, Block LH. Effects of amlodipine on gene expression and extracellular matrix formation in human vascular smooth muscle cells and fibroblasts: implications for vascular protection. *Int J Cardiol* 1997; **62**(Suppl 2): S31–S37.